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**MICROALGAE FROM AEROTERRESTRIAL ENVIRONMENTS:
SCENEDESMUS VACUOLATUS AS A POTENTIAL RESOURCE
FOR MASS CULTIVATION**

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ABSTRACT

Microalgae are promising microorganisms deserving attention within the vast array of new biotechnologies. They are employed in many commercial applications: human and animal nutrition, pharmaceutical and cosmetical components, wastewater treatments.

One of the critical factor for the industrial uses of microalgae is the production of biomass. So two types of cultivation system have been developed to increase the biomass production: liquid cultivations and immobilized cultivations.

Moreover the biomass production depends on the type of organisms. *Scenedesmus vacuolatus* is known to have high biomass productivity among green algae, and has been frequently tested for biotechnological applications.

Understanding the real potentiality of cultivation of this alga still needs new experimental evidences.

In my dissertation, I focus the attention to the possible use of *Scenedesmus vacuolatus* as a model organism for massive cultivation and, consequently, to develop an industrial production, either in liquid- or immobilized-culture systems.

To assess potential applications of *Scenedesmus vacuolatus* in the fields of biomass production, it is necessary to characterize its growth and photosynthetic activity. Different sets of experiments have been carried out in both type of cultivations liquid and immobilized.

Firstly, I analyze several *Scenedesmus vacuolatus* strains and I prove the best candidate for my experiments is 053.

Secondly, in liquid culture, thanks to the support of kinetic model I highlight the bottleneck of the photosynthesis process in photobioreactor in different operating conditions is photoinhibition.

Moreover, in liquid culture, *Scenedesmus vacuolatus* shows a very good growth performances in enclosed photobioreactors achieving high biomass in presence of 2% CO₂.

Thirdly, in the study of immobilized cultivation, thanks to the combination of photograph imaging analysis and pulse amplitude modulation fluorimetry, I study the first steps of the adhesion process of this microalga in submerged condition and analyze its physiological state. Also in this case, *Scenedesmus vacuolatus* shows positive outcomes: it has a good ability of attachment and the adhesion process is scarcely influenced by the kind of carrier used.

Eventually, by studying growth of *Scenedesmus vacuolatus* on a porous substrate bioreactor (the Twin Layer system), the performances of *Scenedesmus vacuolatus* in liquid systems are shown lower than that ones in attached cultivation and also in this case the addition of CO₂ is necessary to increase the productivity. Comparison with data obtained from other organisms in other experiments on porous substrate bioreactor shows that immobilized cultivation of *Scenedesmus vacuolatus* based on Twin-layer system allows to achieve very good values of biomass productivity and light yield and confirms that also in immobilized cultivation *Scenedesmus vacuolatus* is a good candidate for industrial uses.

ABSTRACT

Le microalghe sono promettenti microrganismi che meritano particolare attenzione all'interno della vasta gamma delle biotecnologie. Esse sono impiegate in molte applicazioni commerciali: l'alimentazione umana e animale, prodotti farmaceutici e cosmetici, trattamento delle acque reflue.

Uno dei fattori critici per gli usi industriali delle microalghe è la produzione di biomassa; due tipi di sistemi di coltivazione sono stati sviluppati per aumentare la quantità di biomassa: coltivazioni liquide e coltivazioni immobilizzate.

Inoltre la produzione di biomassa dipende dal tipo di organismi, tra le alghe verdi *Scenedesmus vacuolatus* è noto per avere un'alta produttività ed è stato spesso testato per applicazioni biotecnologiche.

Per capire la vera potenzialità delle colture di microalghe, sono ancora necessario nuove evidenze sperimentali.

Nella mia tesi, ho focalizzato l'attenzione sul possibile impiego di *Scenedesmus vacuolatus* come organismo modello per la coltura di massa e, di conseguenza, di sviluppare una produzione industriale basata su questa microalga, sia in culture liquide che immobilizzate.

Per valutare le potenziali applicazioni di *Scenedesmus vacuolatus* nel campo della produzione di biomassa è necessario determinarne la crescita e l'attività fotosintetica. Diverse serie di esperimenti sono stati condotti per raggiungere questi obiettivi.

In primo luogo ho analizzato diversi ceppi di *Scenedesmus vacuolatus* e ho dimostrato che il miglior candidato per i miei esperimenti è 053.

Poi nelle coltura liquida grazie al supporto del modello cinetico di Eilers e Peters ottimizzato da Gargano et al. ho evidenziare che il maggior problema nel processo di fotosintesi all'interno dei fotobioreattori è la fotoinibizione.

Inoltre ho anche mostrato che nonostante i problemi legati alla fotosintesi in coltura liquida *Scenedesmus vacuolatus*, mostra un ottimo performance di crescita ottenendo elevata quantità di biomassa in presenza del 2% di CO₂.

Nello studio della coltivazione immobilizzato la combinazione di analisi dell'immagine e la fluorimetria PAM si è dimostrato un buon protocollo scientifico per studiare le prime fasi del processo di adesione in condizioni sommerse permettendo di conoscere lo stato fisiologico delle microalghe .

Anche in questo caso *Scenedesmus vacuolatus* mostra risultati positivi: ha una buona capacità di attecchimento e il processo di adesione è scarsamente influenzato dal tipo di substrato utilizzato.

Infine nello studio della crescita di *Scenedesmus vacuolatus* su un bioreattore a substrato poroso (il sistema Twin Layer) le prestazioni di *Scenedesmus vacuolatus* si è mostrato maggiore rispetto a quella nella coltivazione liquida ma anche in questo caso è necessaria l'aggiunta di CO₂ per aumentare la produttività.

Il confronto con i dati ottenuti da altri organismi in altri esperimenti su bioreattori a substrato poroso e i miei risultati ha mostrato che *Scenedesmus vacuolatus* raggiunge ottimi valori di produttività di biomassa e conferma che anche nelle culture immobilizzate *Scenedesmus vacuolatus* è una buona candidato per usi industriali.

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CHAPTER 1

Introduction

1.1 MICROALGAE-BASED BIOTECHNOLOGIES

Microalgae are promising microorganisms which deserve attention within the vast array of new biotechnologies. In ancient times some microalgae such as *Nostoc* and *Spirulina* were used in China as an emergency food to survive during famine (Jensen et al.2001).

However, the study of algological applications has been systematically developed only in the last 40 years, starting in Germany and then in the United States, Japan, Israel, Italy (De la Noue and De Pauwn 1988, Borowitzka et al. 1999).

Nowadays algae are employed in many commercial applications. They are intensively used in human and animal nutrition because they are rich of proteins, carbohydrates and their composition is also comparable with other type of aliments (Becker 2004, Becker 2007, Guil-Guerrero et al. 2004) (table 1).

Table 1. Composition of different human foods and algae (% of dry matter).

Commodity	Protein	Carbohydrate
Bakers' yeast	39	38
Meat	43	1
Milk	26	38
Rice	8	77
Soybean	37	30
<i>Anabaena cylindrica</i>	43-56	25-30
<i>Chlamydomonas reinhardtii</i>	48	17
<i>Chlorella vulgaris</i>	51-58	12-17
<i>Dunaliella salina</i>	57	32
<i>Porphyridium cruentum</i>	28-39	40-57
<i>Scenedesmus obliquus</i>	50-56	10-17
<i>Spirulina maxima</i>	60-71	13-16
<i>Synechococcus sp.</i>	63	15

Moreover, it is possible to extract from microalgae high-added value molecules and pigments such as astaxanthin and phycobiliproteins, that have shown health-promoting properties, and a broad range of pharmaceutical and cosmetical applications (Bermejo et al 2002, Del Campo et al. 2000). Microalgae are also attractive for wastewater treatment because they have the capacity to convert solar energy into useful biomasses, incorporating nutrients such as nitrogen and phosphorus (Sturm and Lamer 2011, Hoffman et al. 1998). Last but not least, microalgal biomass can be converted into energy-rich products. Indeed in comparison to plants, many microalgae strains are extremely rich in oil and fatty acids, which make them potentially useful for the production of biofuel (the oil content of algae may exceed 80% against 5% of the agriculture crops (Rodolfi et al. 2009, Olivieri et al. 2011).

The development of microalgae utilization was also possible thanks to their ability to achieve high concentration of biomass, also when cultured in arid regions, utilizing waters unsuitable for conventional agriculture (Figure 1).



Figure 1. Cultivation of microalgae for industrial products. Algatech Ltd. Israel Kibbutz Ketura.

1.2 MICROALGAE CULTIVATION

The growth pattern of microalgae is characterized by: lag phase; exponential growth phase; phase of declining relative growth; stationary phase; death phase (Becker 2004), and parameter such as pH, temperature, light generally influence the biomass productivity. Three types of cultivation can be performed:

- 1) batch condition where the culture can only complete a limited number of life cycles because new nutrients are not added;
- 2) fedbatch condition where new medium is added whenever it is necessary to prevent the depletion of nutrient;
- 3) semicontinuous condition where a sample of fixed volume is removed at regular time intervals and the same quantity of new medium is added.

1.2.1 LIQUID CULTURES – open and closed systems

Since the 1950 cultivation in open pond production systems has been used (Brennan and Owende 2010). There are different forms of open pond cultivation: natural or artificial ponds, race-track ponds, and cascades (Figure 2) (Masojídek and Torzillo 2008).

Open culture systems require large surface areas exposed to the surroundings, allowing for natural illumination and gaseous transfer between the algal culture and the environment. (Christi et al. 2007). Moreover they are shallow to allow maximum sunlight penetration (Murthy 2011).

These systems are the simplest method of algal cultivation and offer a lot of advantages such as simple processing and low costs. Furthermore, the culture is mixed to prevent the cells from sedimentation at the bottom as well as to distribute efficiently cell growth at the sunlight.

However, open ponds have also a lot of disadvantages. Firstly, it is impossible to control light and temperature which depend only on the location of the facility. Furthermore, it is extremely hard to maintain algae monocultures in such open pond systems and avoid the contamination with other algae or other microorganisms.

Photobioreactors are the typical closed systems and they permit the swap of light and energy but exclude material exchange with the surrounding.

One of the most important characteristic of the closed system is the mixing that prevents the sedimentation of biomass and thermal stratification (Molina-Grima et al. 2004).

Moreover, compared to the open systems, photobioreactors allow to optimize parameter according to the biological and physiological characteristics of the microalgae.

So photobioreactors are characterized by several advantages: low contamination; no water loss; high biomass productivity at high cell density; harvesting costs reduced with respect to the open system; possibility to grow a wide spectrum of species by tuning the operating conditions.

However closed systems present several disadvantages such as the photo-oxidative damage of the cells and the increase of pH. Indeed the oxygen produced during the photosynthesis may accumulate and inhibits the growth of the cells and the production of chlorophyll. On the other side, the CO₂ uptake is associated to the pH increase and inhibits the growth of the cells (Camacho Rubio et al. 1999). Furthermore the costs of photobioreactors are more higher than that ones for open ponds, especially the operating costs due to the higher energy required for water circulation and to stabilize temperature, and the costs of maintenance (they are more difficult to clean) (Weissman and Benemann 1998).

There are different types of photobioreactors, generally classified according geometry. *e.g.* tubular; flat; horizontal; inclined; vertical; spiral (Ugwu et al. 2008) (figure 3).



Figure 2. Photobioreactor and open system.

1.2.2 IMOBILIZED CULTIVATIONS: biofilms and their applications

The aggregation of microorganisms that not live in status of isolated single cell but form a community growing on solid surfaces enclosed in a matrix of extracellular polymeric substances is called biofilm (Flemming and Wingender 2010).

Different microorganisms have the ability to create a biofilm. Among these, there are aeroterrestrial microalgae, that show a peculiar ability to adapt themselves to the harsh conditions of terrestrial habitats (Menicucci 2010). Aeroterrestrial microalgae typically colonize the interface between all kinds of hard substrata and the atmosphere. The surface can be of natural origin, e.g., tree barks, hairs, soil, and rocks, or of artificial origin, e.g., roof tiles, or building facades in urban areas (Figure 3), creating aesthetical and structural damages such as discoloration of artwork and biodeterioration of historical building (Ettl 1995, Ortega-Calvo et al. 1995, Gaylarde and Morton 1999, Häubner et al. 2006).



Figure 3. Biofilm in presence of natural substrate and manmade substrate.

The aeroterrestrial microalgae are considered a first choice for the selection of strains for mass cultures because they are particularly resistant to different kinds of stresses. For example, aeroterrestrial microalgae have the ability to survive to desiccation for a long period also when the reproduction is stopped (Holzinger and Lutz 2011).

Stichococcus species and *Chlorella luteoviridis* recover photosynthesis only after few minutes then moisturizing (Häubner et al. 2006), whereas the growth of many aquatic microalgae, such as *Nannochloropsis* species, is significantly limited by desiccation. Furthermore aeroterrestrial microalgae are highly resistant to other hostile environmental conditions such as low temperature and variations in salinity, UV (Lukesová et al. 2008). As previously described, the most common approach in algae cultivation is the liquid method.

In these systems microalgae are diluted: generally around 99% of culture volume consists of water and only remaining 1% is the dry algal biomass (Leher and Posten 2009). In the

last years, to enhance biomass production, new types of photobioreactors (PSBRs) based on immobilized microalgae have been developed (Schulze et al. 2015).

The core principles of the immobilized method include (Liu et al.2013):

- 1) algal cells are immobilized onto a supporting material to form a layer of algal population;
- 2) multiple layers of these films receive the diluted sunlight;
- 3) a volume of culture medium is supplied to the supporting matrix materials to provide nutrients and moisture to the attached algal cells for growth.

The immobilized technique of cultivation can be commercially more feasible than ordinary liquid cultivation method, and yielding a higher biomass (table 2). In addition, thanks to the porous substrate bioreactors, it is much easier to separate microalgae biomass from the medium (Cheng et al. 2013 Ledwock et al. 2015), and also the harvesting is simpler: algae are scratched and dried.

Table 2. Productivity comparison of suspended and non-suspended cultivation (from Ledwock et al. 2015).

Species	Attached cultivation productivity [g/m ² per day]	Suspended cultivation productivity [g/m ² per day]	Reference
<i>Scenedesmus obliquus</i>	70.9	8.9–14	Liu et al. 2013
<i>Botryococcus braunii</i>	5.5–5.7	2.4	Liu et al. 2013 Cheng et al. 2013

The characteristic of the surface play an important role for the adhesion of microalgae to substrate. Generally, microalgae grow better on rough and hydrophobic materials (Köhlner et al. 1999, Cowling et al. 2000, Verran et al. 1991). Furthermore, also the sterilization of the surface influences the process of attachment (Djemai-Zoghache et al. 2011). Several studies confirm that cotton is the best material for growing microalgae biofilm, it would seem more effective than nylon, polypropylene, acrylic, or jute and is one of more economics, too (Gross et al.2013, Christenson and Sims 2012) .

The porous substrate bioreactor is based on a new technology and there are few available studies on this subject, compared with the large body of knowledge available for liquid

systems of cultivation. There are different models of porous substrate photobioreactor (figure 4).

In the rotating cultivation system, the porous substrate bioreactor is partially immersed in liquid medium. A rotation of reactor permits the biomass to alternate the presence in liquid rich in nutrients and the presence in atmosphere with higher concentration of carbon dioxide (Gross et al. 2013, Christenson and Sims 2012).

In the twin layer system, microalgae are attached by self-adhesion on a hydrophilic, sheet-like porous substrate, which divides algal biomass from the bulk of the medium (Melkonian et al. 2010, Nowac et al. 2005).

In the attached cultivation system, a surface with attachment material covered by a layer of filter paper is inserted on a plate vertically oriented. The immobilized microalgae are submerged in a reservoir where liquid is forced to circulate by a pump and mixed with bubbling air (Liu et al. 2013).

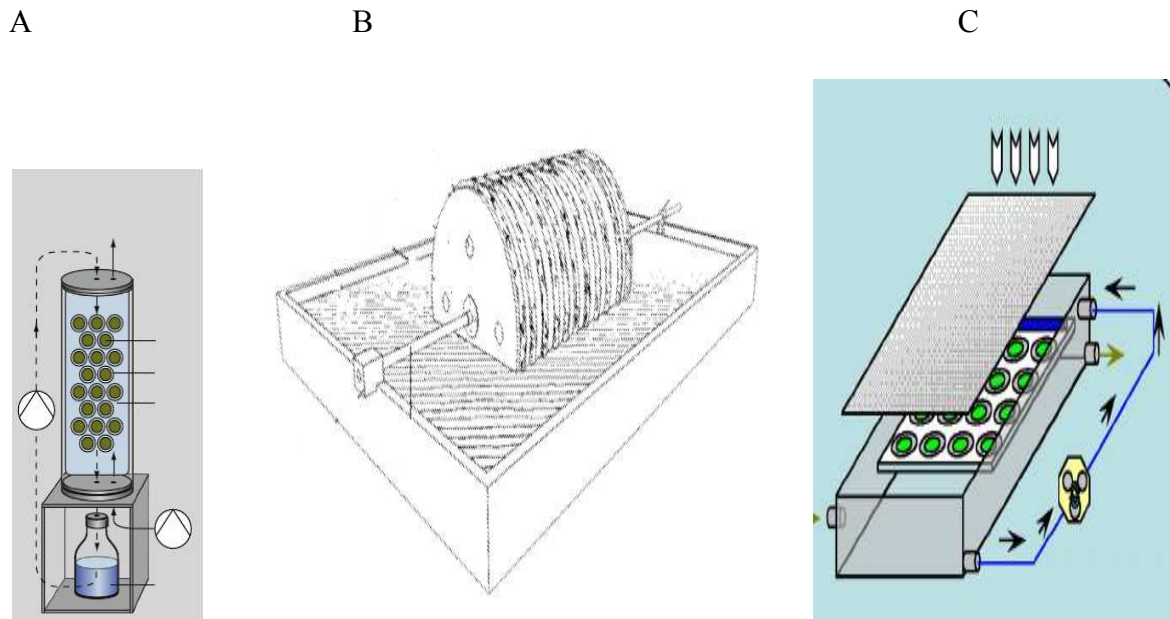
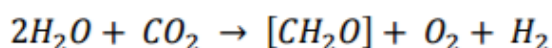


Figure 4. Three types of porous substrate bioreactors. A twin layer, B rotating cultivation system, C attached cultivation.

1.3 PHOTOSYNTHESIS AND LIGHT UTILIZATION

The evolution of life forms on the Earth depends on photosynthesis. This mechanism represents a unique process of sunlight energy conversion. The algal biomass production is directly proportional to the efficiency with which the algal cells assimilate carbon dioxide from the atmosphere through photosynthesis.

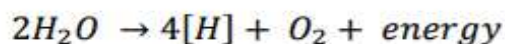
The equation of photosynthesis can be briefly represented as:



This process is very complicated and not all details are known. Photosynthesis takes place in chloroplasts and in particular thylakoid membranes (Sener et al. 2007). It is divided in two reaction stages: light reactions and dark reactions (Crill 1977).

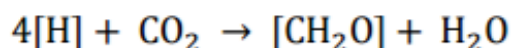
In the light reactions, photons from sunlight are absorbed by chlorophyll-containing proteins. Subsequently there is a set of photochemical-redox processes which provide energy such as ATP and NADPH.

The final equation of reactions is:



In the dark reactions, called also Calvin-Benson Cycle, CO₂ is captured by RuBisCO (Ribulose-1,5-bisphosphate carboxylase oxygenase), thanks to the presence of ATP and NADPH produced in the light reaction period. CO₂ is used to produce organic matters. (Masojidek et al. 2004).

The final equation of this step is:



It is possible to study photosynthesis thanks to two non invasive methods: gas exchange and photochemical process.

The gas exchanges allow to analyze the rate of photosynthesis by net photosynthetic oxygen evolution (O₂ evolution) and dark respiration rates (O₂ uptake). Indeed gross photosynthesis is considered as the sum of these two factors (Glud et al. 2000). Generally at low irradiance, the rate depends linearly on light intensity, whereas at high light intensity, when enzymatic reactions utilizing fixed energy become rate limiting, photosynthesis achieves a plateau. Under prolonged highest irradiance, photosynthetic rate

usually declines from this light-saturated value (figure 5). This phenomenon is commonly called as photoinhibition of photosynthesis (Hall and Rao 1999).

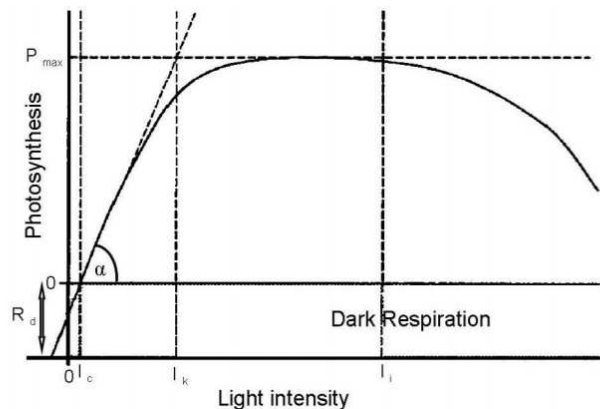


Figure 5. Photosynthetic light-response curve. The initial slope α is the P_{max}/I_k ratio, where I_k is the saturation irradiance and P_{max} the maximum rate of photosynthesis. R_d is the rate of dark respiration.

The data of gas exchange are generally integrated with fluorescence data. Generally light energy absorbed by chlorophyll molecules has three fates:

- 1) it is used for photosynthesis;
- 2) it is dissipated as heat;
- 3) it is reemitted as light chlorophyll fluorescence.

These three processes are in competition: when the efficiency of one of these increases, the yield of two others decreases (Maxwell et al. 2000). Hence, by measuring the yield of chlorophyll fluorescence, information about changes in the efficiency of photochemistry and heat dissipation can be gained.

Indeed under light conditions, photosynthetic reaction centres of photosystem 2 (PSII) are in an excited state. The energy of excited state is used for photochemical process. Alternatively, the excitation energy can be dissipated as heat, or re-emitted as fluorescence. Different kinetic models have been developed to describe photosynthesis and help to resolve the pitfalls of this process.

They can be divided into two groups (Bernardi et al. 2014):

- 1) physiological models based on the interaction between photosynthetic mechanisms and nutrient conditions (Geider et al. 1998; Marshall et al. 2000);
- 2) the state models based on the relationship between light intensity and rate of photosynthesis (Eilers and Peeters 1988, Garcia-Camacho et al. 2012), taking into

account different factors, such as photoadaptation, photoinhibition, and “flashing light” effect.

The biomass productivity is linked to the ability to utilize the available solar radiation driving photosynthesis. So, it is important to consider two phenomena to optimize biomass productivity: photoadaptation and photoinhibition, that have been intensively studied in microalgae, mainly under laboratory conditions (Powles et al. 1984).

Photoadaptation is the capacity of eukaryotic algae to adapt light harvesting ability to fluctuation in ambient light regime. Indeed, microalgae show the ability to capture and receive light energy modifying various processes such as onset of gene expression, metabolic and biochemical pathways, assembly and synthesis of building blocks for special sub-cellular structure (Bennett and Bogorad, 1973).

Photoinhibition is the light-induced reduction of photosynthetic capacity of microalgae. Generally, this phenomenon occurs when level of light intensity exceeds the saturation level, where the maximum growth is obtained (Ogbanna 2000). Photoinhibition has deleterious effect on productivity of microalgae culture and leads to the reduction of cells population.

1.4 *SCENEDESMUS* PHYLOGENESIS AND MORPHOLOGY

The genus *Scenedesmus*, instituted by Meyen (1928), belongs to the family Scenedesmaceae (Oltsmanns, 1904), in turn divided into six subfamilies (Komarek and Fott, 1983). In particular *Scenedesmus* belongs to the subfamily of Scenedesmoideae. Following the description of Komarek and Fott (1983) the genus *Scenedesmus* is known to be made up of 2-32 cells flat cenobia. This is an aggregation of four or eight cells inside a parental mother wall and can have several architectures: linear, costulatoid, irregular, dactylococcoid (Lüring M. et al. 1999). The formation of coenobia depends on several factors such as grazing and nutrient-depleted conditions.

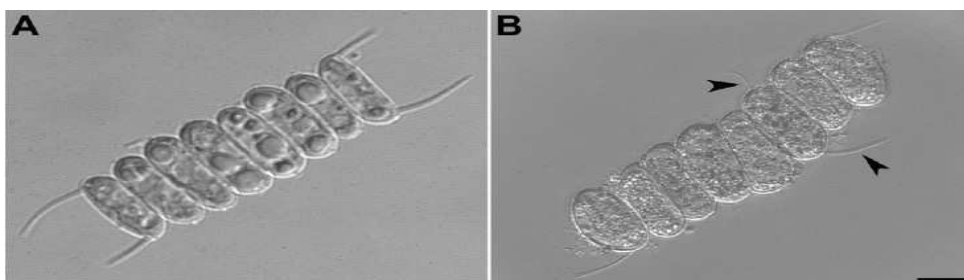


Figure 6. Microphotographs of eight-celled coenobia of *Scenedesmus quadricauda*.

The cells may have different shapes, but they are always elongated, with the poles from truncated acute to obtuse. The cell wall can be smooth or with grooves of different type, with or without spines. The chloroplast is single, with one pyrenoid. Reproduction by autospore formation, which, even in the mother cell, are organized to form a new cenobium. During replication, the mother cell becomes larger and multinucleated, after multiple nuclear divisions. The cytoplasm is divided into uninucleated daughter cells which typically form a colony inside the parental cell wall before being liberated (Pickett-Heaps et al. 1975).

Over 800 taxa have been attributed to the genus *Scenedesmus* (Hegewald and Silva, 1988). Although it has also been described a phase of the life cycle in which they produced zoospores (Trainor, 1993), this phenomenon has been observed so rarely, both in nature and in laboratory cultures, to be considered of little importance.

Following the taxonomic treatment proposed by Hegewald (1978), *Scenedesmus* can be divided into three sub-genera, such *Acutodesmus* Hegewald, spindle cells with acute poles and smooth cell walls, *Scenedesmus*, cells with poles truncated/obtuse and smooth cell walls, *Desmodesmus* Chodat, cells with poles truncated / obtuse, and the walls with grooves of various kinds and, occasionally, thorns.

Acutodesmus

The taxonomy of this subgenus is mainly based on the cell shape and arrangement of the cells in the cenobia. Frequently the cenobia of the species included in *Acutodesmus* disintegrate, forming cell populations consisting of single cells, especially when the culture conditions are optimal. In this subgenus they are also present species that possess thin filamentous structures consisting of hemicellulose, placed externally to the cell wall.

Scenedesmus

At this subgenus belong all the species with obtuse cellular poles, without thorns and grooves of outer layers wall.

Desmodesmus

All species that belong to *Desmodesmus* possess an outer layer of the wall consisting in turn by 4 (and not 3, as in other subgenera) layers of sporopollenin. The outermost layer of sporopollenin produces the structures called grooves, visible under an optical microscope as granules or as streaks. Most species also possesses spines of different lengths. The

grooves can be observed only with the electron microscope. It is possible to distinguish three types of grooves:

1) Warts They are formed by the folding of the outer layer of Sporopollenin and are empty tubular structures;

2) Tubes. Also tubular like predecessors, grouped in bundles, forming strips running along the surface of the cell (section Armatus);

3) Rosettes. A basal bristle producing structures resembling studs. Rosettes are ring-shaped structures enclosing little piles on the cell surface (Stahelin et al. 1975).

However, it should be noted that the morphology of the various *Scenedesmus* species is strongly influenced by the culture conditions, for which the same strain, in the presence of sufficient nutrients may develop a certain morphology, which changes significantly (cenobia converting to single cells, rearrangement of the external layers of the wall, production of very different length spines, and so on) if experienced scarcity of some specific nutrient.

The polymorphism of the genus leads to erroneous identifications. *Scenedesmus* a typical aeroterrestrial genus inhabiting soils, rocks and on other substrates, such as tree bark, and very frequently the species when are observed in the field generally appear as unicellular, and are often mistakenly regarded as taxa belonging to the genus *Chlorella*.

This is also the case of *Chlorella emersonii* Shihira et Krauss. The species was established in 1965 with the following diagnosis:

Chlorella emersonii spec. Nov.

Cells spherical or elliptical, 4-16 μM in diameter, closer to 16 μM when grown on culture media enriched with glucose. Chloroplast reticulate, green; It tends to brown with aging or shortage of nitrogen. Pyrenoid always present. Good growth rate on agar. It grows well on inorganic culture media and light. The glucose strongly stimulates the growth in autotrophy, as mannose and fructose, that also stimulate the growth in the dark.

Saccharose, however, improves the growth rate only in the light. Acetate is slightly inhibitory in autotrophy and does not support the growth in cultures kept in the dark.

Nitrate and ammonium are nitrogen sources used by the alga the same way. As a nitrogen source, casein hydrolyzate supports the growth best of nitrate. The yeast extract does not promote to the growth of the alga; if administered with glucose, is slightly inhibitory. The

growth under heterotrophic conditions is however poor, and ends after 3-4 days, independently of any organic substrate used.

Shihira and Krauss (1965) instituted in the same paper another species, closely related to *C. emersonii*:

Cells usually elliptical in inorganic liquid culture, long 4.5-8 μm and wide 3.5-7 μm ; only the largest cells tend to be spherical in the inorganic culture media. If grown on culture media with glucose the majority of the cells tends to be spherical, with a diameter of about 8 μm . The chloroplast, in the form of belt or cup, is green to brown with age, or if cells are grown in nitrogen deficient media. Pyrenoid present. Good growth on agar. It grows well in inorganic media exposed to light. Glucose always stimulates the growth, both with the light and in the dark. In autotrophic cultures galactose, mannose and fructose stimulate growth. In the dark galactose as well as mannose and fructose mildly stimulates growth. Other sugars are ineffective. The acetate helps the growth in the light, but not in the dark. As a nitrogen source ammonium is slightly better than nitrate, while casein hydrolyzate is equal to the nitrate. The yeast extract stimulates the growth on glucose, but it is inhibitory when casein hydrolyzed is added to glucose.

In describing this new species, the authors commented how it was very similar to *C. emersonii*. In addition, they instituted some varieties of *C. fusca* including *C. fusca* var. *vacuolata*:

Chlorella fusca var. *vacuolata*

cells elliptic in inorganic liquid culture, long 5.5-12 μm and wide 3.5-11 μm ; the largest cells tend to be spherical in inorganic culture media; if grown on culture media with glucose the majority of the cells tends to be spherical, with a diameter of about 12 μm . Chloroplast reticulate; green and brownish with age or if grown in nitrogen-deficient cultures. Pyrenoid present. Good growth on agar. It grows well in inorganic soils exposed to light. The glucose always stimulates the growth, both in the light in the dark. Galactose, mannose and fructose stimulate the growth in the light, and the last two very weakly even in the dark, while the sucrose does not contribute to improve the growth in cultures exposed to light. As nitrogen sources ammonium, nitrate and casein hydrolyzate gave the same results. The yeast extract and thiamine are ineffective.

Fott & Novakova (1969) on the basis of morphological observations, considered synonyms *Chlorella emersonii* and *C. fusca*, retaining the latter combination. Based on molecular analysis, *C. fusca* var. *vacuolata* was later transferred to the genus *Scenedesmus*, assuming the binomial *S. vacuolatus* (Hegewald, 1997). However, Its position within the

taxonomical framework proposed by Hegewald (1978) remains undefined, being located halfway between subgenera *Scenedesmus* and *Desmodesmus* (Hegewald, 1997).

1.5 POTENTIAL APPLICATION OF *SCENEDESMUS*

The genus *Scenedesmus* is known to have high biomass productivity among green algae, and has been frequently tested for biotechnological applications.

Some species are resulted particularly interesting for the possible industrial uses.

Scenedesmus obliquus is considered one of the better candidates for the production of bioenergy such as bioethanol; indeed its biomass is rich of carbohydrates and, after hydroxylation with 2% sulfuric acid, achieves a very high yield (Ho et al. 2013). *S. obliquus* is also interesting for the production of another form of energy: biodiesel.

In some particular conditions such as heterotrophy and nitrate depletions, this microalga increases lipids productions (Mandotra et al. 2014). The most significant improvement in lipids production is obtained when stationary phase cultures are transferred to a medium deficient in nitrate for 7 days and phosphate for 3 days, respectively (Mandal et al. 2009).

Scenedesmus acutus is considered important specially for wastewater treatments. It has shown significant results in bioaccumulation of heavy metals for example chromium at high light intensity (Gorbi et al. 2001) and also cadmium in several strains (Canizares-Villanueva et al. 2001).

Moreover this organism has the ability to grow and actively deplete eutrophicating inorganic molecules present in wastewater (González et al. 1997). It has been noted a complete nitrogen removal coupled with a biomass production in strain growth in a photobioreactor filled with wastewater collected from the final step of the local urban purifier plant containing nitrate (Doria et al. 2012).

Scenedesmus vacuolatus, has been successfully employed in the biotransformation of sterols, being able to catalyze reactions of hydroxylation, reduction and side chain-degradation (Della Greca et al. 1996, Pollio et al. 1996), and has recently revealed a promising cosmeceutical prospective (Chatzikonstantinou et al. 2016). Moreover photosynthesis and glycoliate metabolism of *Scenedesmus vacuolatus* cells immobilized in alginate beads remain unchanged over a 6 month period (Day and Codd 1985); immobilized cells of this species are able to sequester both heavy metals (Wilkinson et al., 1990) and chloride compounds (Zhang et al. 1998) from wastewaters. For these

reasons, *S. vacuolatus* can be considered as an ideal candidate for immobilized culture systems.

AIM OF THE THESIS

To understand the real potentiality of cultivation and processing of microalgae cultures, it is still necessary to produce new experimental evidences. Scenarios where systems can be implemented to develop industrial production are extremely uncertain, and it is unlikely to have one single best solution.

In my dissertation, I have focused the attention to the possible use of *Scenedesmus vacuolatus* as a model organism for massive cultivation and, consequently, to develop an industrial production based on this microalga, either in liquid- or immobilized-culture systems.

To assess potential applications of *Scenedesmus vacuolatus* in the fields of biomass production it is necessary to characterize its growth and photosynthetic activity. Different sets of experiments have been carried out to achieve this goal.

Preliminarily, I have screened several *Scenedesmus vacuolatus* strains, evaluating the growth rate in presence of different conditions, their ability to remove phenols and produce lipids. On the basis of the obtained results, the strain *Scenedesmus vacuolatus* 053, was selected for the subsequent experiments (chapter two) .

Then, through the use of a model, I have investigated the effect of the most common operating conditions used in massive microalgae liquid cultivations on the performance of photochemical process . Then results of the photochemical process efficiency have been integrated with gas exchange analysis. By this approach, it is possible to assess the optimal values of light intensity maximizing the photosynthesis rate (and, consequently, increasing the biomass productivity)(chapter three).

The second part of the thesis is focused on attached cultivations. This state generally allows to achieve high biomass concentration. The adhesion to substrate is the critical phase in the formation of biofilms. Therefore, firstly I have evaluated the growth and photosynthetic activity during the first steps of the microalgae adhesion process on different substrates by studying photochemical process and with help of photograph imaging analysis (chapter four). Finally, I have measured biomass productivity and

pigment concentrations of microalgal biofilm growing on a solid photobioreactor, the twin-layer system. Eventually, results obtained in attached cultivation have been compared to that ones in liquid culture(chapter five).

CHAPTER TWO

***Scenedesmus vacuolatus* cultures for combined phenoloxidase activity and biodiesel production**

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Abstract

A key aspect for the industrial development of microalgal-based high value by-products production processes is the cost fixed and operating contributions. A potential solution is the development of microalgal-based biorefineries coupling the production of biofuels to services and products. The coupling of both biofuel production and wastewater treatment has been proposed as a challenging biorefinery. *Scenedesmus vacuolatus* was selected for the high performances of extracellular phenoloxidase that could improve the phycoremediation of hazardous phenolic pollutants. On the other hand, *Scenedesmus* species resulted also interesting candidates for biofuel production.

The present contribution reports results of a research activity regarding the selection of the most promising *S. vacuolatus* strain, in terms of growth rate at different pH, salinity and glucose concentrations, and the assessment of the optimal conditions for *S. vacuolatus* cultures aiming at a multifold target: efficient nitrogen removal, high lipid production, and enhanced release of extracellular phenoloxidase in the medium.

Photobioreactors performances were reported in terms of biomass and lipid productivity, final biomass concentration, maximum phenoloxidase activity, and liquid phase features. Lipid composition of microalgae were also analysed after extraction and alkaline transesterification. Results showed that: C/N ratio in liquid culture resulted of about 3.8, near to that of protein; biomass productivity doubled when photobioreactors were sparged with 5% CO₂ supplemented air with respect to cultures sparged with air; N-starvation did not improve the lipid/biomass ratio but it enhanced the transesterified fraction of lipids; phenoloxidase activity was completely suppressed by presence of 5% CO₂ in the gas phase; the lack (suppression) of phenoloxidase activity did not affect algae growth or lipids biosynthesis.

1. Introduction

Phenols and their derivatives are a class of chemical compounds in which the aromatic hydrocarbon group is bonded directly to an hydroxyl group (-OH). They are the major components of dyes, polymers, drugs, resin paint and other organic substances (Michalowicz and Duda, 2007; Gad and Saad, 2008). Their presence in the environment is mainly related with production and degradation of numerous pesticides, plastics, adhesives, iron, steel, aluminum and leather, and with the generation of industrial and municipal sewages (Fleeger et al., 2003).

Phenols are harmful exotoxins: upon contact with the skin and eyes they induce their irritation, when inhaled, they can be cause of comas, convulsions and cyanosis, internally, they can affect the liver, kidneys, lungs, and vascular system (Busca et al., 2008). Gad and Saad, 2008, reported that phenols can even induce mutagenesis and carcinogenesis due to their relevant peroxidative capacity. Toxic action of phenols can be related with two major causes: hydrophobicity of the phenolic compound involved and the generation of organic and free species of radicals and reactive oxygen species (Wasi et al., 2013;).

Since phenolic compound is found in several factories effluent, each country have been established their own laws and acts to regulate the level of phenols in the air and in the effluent and water bodies (Alkhuraiji et al., 2017). U.S. Environmental Protection Agency (US EPA) and World Health Organization (WHO) limited the phenol concentration at less than 1 µg/L in drinking waters and 1 mg/L in wastewater (Dabhade et al., 2009). European Drinking Water Directive 98/83/EC, 1998, set up a limit of 0.5 µg/L in drinking water and 0.1 mg/L in industrial effluent (Tziotzios et al., 2005; Al Zarooni and

Elshorbagy, 2006), while Japan and Argentina limited the phenol concentration in water source at 5 mg/L and 2 µg/L, respectively (JEGS 2012; Coniglio et al., 2008).

The methods mainly used to remove phenols in contaminated water or wastewater can be largely categorized into three types: physical, chemical, and biological. Those methods can be used separately or in combination in dependence of type and extent of contamination (Si et al., 2013).

Biotechnological techniques, such as phycoremediation, are becoming attractive methodologies for phenols removing from wastewater. Phycoremediation, investigated since 1960s (Oswald and Gotaas, 1955; Allen, 1968) consist in the use of plants, macroalgae and microalgae for the removal or biotransformation of industrial and municipal wastewaters, including heavy metals, hydrocarbons and xenobiotics from wastewater and CO₂ from waste air (Allen, 1968; Podder and Majumder, 2017). Ajayan et al., 2015, and Pacheco et al., 2015, described the phycoremediation as a non-conventional wastewater treatment because it is noninvasive, lucrative, efficient, safe, and environmental friendly process. The results of the decontamination process by microalgae is the biomass production (Rawat et al., 2011).

Microalgae are photoautotrophic microorganism that can be found typically in freshwater and marine systems. They are unicellular organism that can be grow individually, in chains or groups. They easily adapted to the environment and are able to grow in almost all environments, tolerating various environmental conditions (Pacheco et al., 2015). The microalgal biomass can be used for several applications including aquaculture, human nutrition, animal nutrition, pharmaceutical, bioenergy and other valuable chemicals production (Yen et al., 2013). Several microalgae species were tested to evaluate their capacity to remove pollutants in wastewater and their potential in the phycoremediation process. The most reported potential species are *Chlamydomonas reinhardtii*, *Euglena pascheri*, *Lyngbya pseudospirulina*, *Botryococcus braunii*, *Azospirillum brasilense* and *Scenedesmus* sp. (Kong et al., 2010; Pacheco et al., 2015; Ajayan et al., 2015;; Podder and Majumder, 2017; Paskuliakova et al., 2016; Escapa et al., 2016).

Algae belonging to the genus *Scenedesmus* have demonstrated great ability to degrade phenols and their derivatives (Pollio et al., 1993; Pinto et al., 2002; Pinto et al., 2003; Jais et al., 2015; Jàcome-Pilco et al., 2009; Zhou et al., 2012). Pinto et al., 2002, 2003 pointed out that in dark condition, *Scenedesmus quadricauda* can degrade over 50% of the phenols contained in olive oil mill wastewater, however the phenols were not completely removed, but bio-transformed into other aromatic compounds. Jais et al., 2015, pointed out that

Scenedesmus sp can remove the 74.77% of Nitrogen, 82.17% of Phosphorus, 86.36% of total organic carbons, 65.76% of Iron and 84.14% of Zinc from wastewater. Jàcome-Pilco et al., 2009, revealed that *S. incrassatus* can remove Cr^{VI} with an efficiency of 43.5±1.0%. Moreover Zhou et al., 2012, proved that *S. obliquus* is able to remove Zinc and Copper from aqueous solutions with an efficiency close to 100%.

However, a negative aspect for the industrial development of microalgal-based plant is the cost: fixed and operating contributions. Therefore, to increase the profitability and the sustainability of the microalgal-based industries, the coupling of the wastewater-algae treatment with the bio-energy production has long been proposed (Hena et al., 2015;), and *Scenedesmus* has been frequently proposed as a possible candidate to produce biodiesel from microalgae (Griffiths et al., 2010; Mata et al., 2010).

In the present work the undertaken aims were to evaluate the possibility to use *Scenedesmus vacuolatus* cultures for simultaneous removing of phenols by releasing phenoloxidase in the culture medium and producing lipids for energy production. A biotechnological approach was applied to enhance the phenoloxidase activity in the medium and to improve the phycoremediation of hazardous phenolic pollutants.

2. Materials and methods

2.1. Microorganism and medium

Scenedesmus vacuolatus (Chlorophyta), strains 53, 54,61, 235, 315, 316, 317 were from the algal collection at the Department of Biology of the University “Federico II” of Naples (ACUF) (<http://www.acuf.net>). Bold Basal Medium (BBM), supplemented with NaNO₃ - 40 mg/L - as nitrogen source, was used. BBM was autoclaved for 20 min at 120°C. The pH of the autoclaved medium was about 7. Glycylglycine and NH₄Cl were supplemented to BBM as buffer for tests carried out at pH=3.0 and 8.5, respectively. HCl and NaOH were used to adjust the pH. HCl and NaOH were used to adjust the pH. NaCl and Glucose were added to BBM cultures to have final concentrations of 0,25, 0,50, 1, 2, and 3% for NaCl, and 0.25, 0,50 and 1% for glucose.

2.2. Operating conditions and procedure

Vertical bubble columns (VBC) were used (Olivieri et al., 2013) for microalgal growth. The VBC was a 1 L cylinder (0.04 m ID, 0.80 m high) with an operating volume of 600 mL. Gas stream was sparged at the bottom of the photobioreactor by means of a porous ceramic diffuser; the irradiance was continuous and it was set at 140 µE/m² s). The head of photobioreactors was equipped with three ports for gas inlet, gas outlet and sampling

operations. The photobioreactors were housed in climate chambers (M2M engineering) equipped with lamps either at the wall. Temperature in climate chambers was set at 23 ± 1 °C. The volumetric flow rate of the gas stream - sterilized through a $0.22 \mu\text{m}$ filter - was set at 20 nL/h. A gas mixing device (M2M engineering) provided the mixing of pure CO_2 in the gas stream with a CO_2 concentration equal to 5%. Biomass concentration (X) was estimated as optical density at 600 nm with a spectrophotometer (Specord 50 – Analytic Jena).

Photobioreactors were inoculated with 1/10 of the final working volume. Tests were carried out in three different phase with respect to the liquid phase: batch, fed-batch and semi-continuous. Under fed-batch conditions a concentrated BBM (10 times the concentration of medium 1X) was supplemented to the photobioreactors when the TN was lower than 10 mg L⁻¹. The volume of concentrated BBM was 1/10 of initial culture volume and it almost balanced the liquid losses by sampling and water evaporation. Under semi-continuous operations 35% of microalgal suspension was weekly replaced with fresh medium. Therefore the average dilution rate (D) – assessed as the ratio between the weekly-replaced suspension volume (F) and the photobioreactor working volume (V) – was 0.05 d⁻¹. Steady state conditions were typically approached in about one month. The sampling operation took place two or three times a week. Data at steady state conditions were calculated as the average value: biomass concentration ($X_{\text{steady state}}$), volumetric productivity (PX), areal productivity (AX), photon yield ($Y_{X/E}$), lipid percentage (%Lipid) and FAME percentage (% FAME).

2.3. Liquid phase characterization

Liquid phase was characterized in terms of pH by a pHmeter (Consort R305); total nitrogen (TN) and total inorganic carbon (IC) concentrations, in the liquid phase, were measured by means of a TOC-V CSH analyzer (SHIMADZU).

The presence, in the culture medium, of phenoloxidase was estimate by biochemical assay, in the presence of 2,2'-azino-bis (3-ethylbenzotiazolin-6-sulfonic acid) (ABTS) (Li et al., 1999; Johannes and Majcheresky, 2000). The phenoloxidase catalyze the oxidation of the ABTS to ABTS^+ in McIlvaine buffer solution. The speed of oxidation was assayed at 25°C and was monitored in a spectrophotometer at 420 nm; the results were expressed in the enzymatic units per mL of supernatant (U/mL) (Piscitelli et al., 2005). The increase of ABTS^+ concentration is directly proportional to the enzymatic activity.

2.4. Biomass characterization

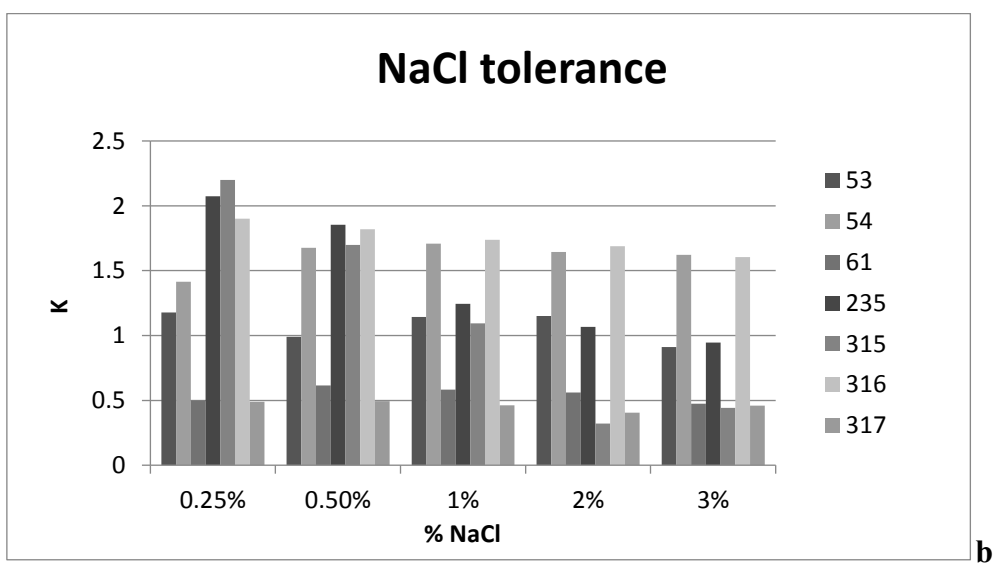
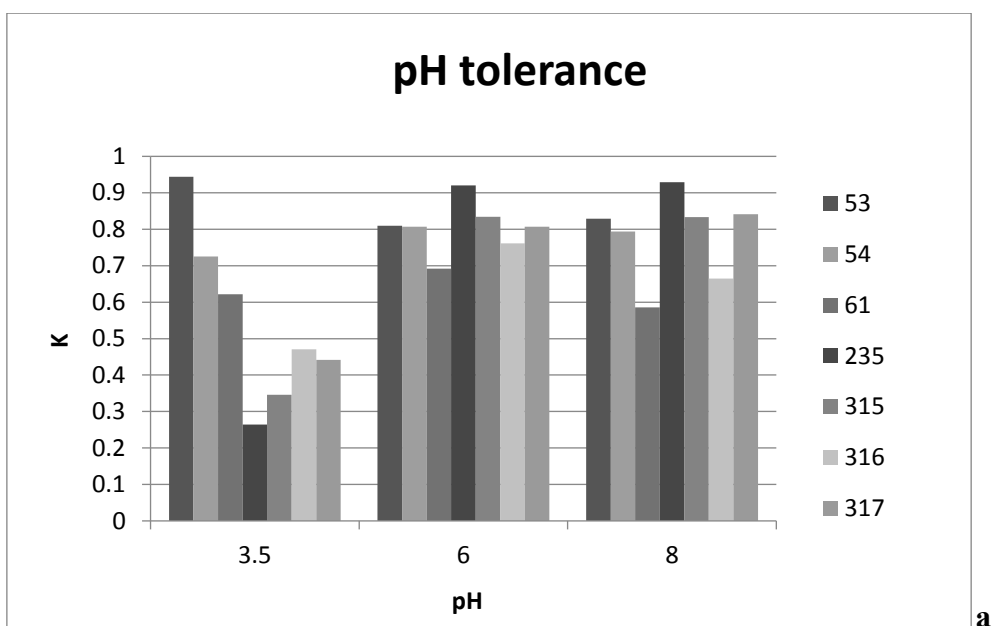
The procedure for the analysis of the microalgae lipid content was: I) biomass harvesting by centrifugation for 20 minutes, 5000 rpm at 5 °C (Eppendorf-5804 R); II) biomass freeze-drying at -50°C (LabconcoFreezon); III) lipid extraction with a 2:1 chloroform-methanol solvent mixture in a Soxhlet apparatus for 8 h; IV) lipid transesterification with methanol and 1.5 %w NaOH at 65 °C for 3 min; V) methyl esters analysis through HPLC (Agilent 1100) (mobile phase: water and acetonitrile; column: Synergy 4u; detector: UV/Vis).

3. Results

3.1. Screening of *S. vacuolatus* strains

The ecophysiological features of *Scenedesmus vacuolatus* strains are presented in Figure 1 (a-c). All the strains were able to grow in the investigated pH range (3.5.-8.0), but some were less tolerant to low pH, particularly the strain 235. The strain 53 showed the highest growth rate at low pH, whereas the strain 315 exhibited the best growth at pH 6.0 and 8.0. NaCl concentrations from 0.25% to 1% stimulate the growth rate of the majority of strains, whereas, some strains, particularly 61, 315, and 317 were sensitive to 2 % and 3% NaCl, whereas the strain 316 showed the highest tolerance to the selected range of salt concentration. Finally, glucose did not stimulate the growth rate of most strains. Only for the strains 53 and 54 were found values of the growth rate slightly higher than those observed without the addition of organic substrates in the culture medium.

The analysis of the results of screening suggests that *S. vacuolatus* strains present homogeneous characters. The conditions of the following tests dictate the selection of strains 53 and 316 that respectively showed the best performances of growth in the range of pH and salinity tested.



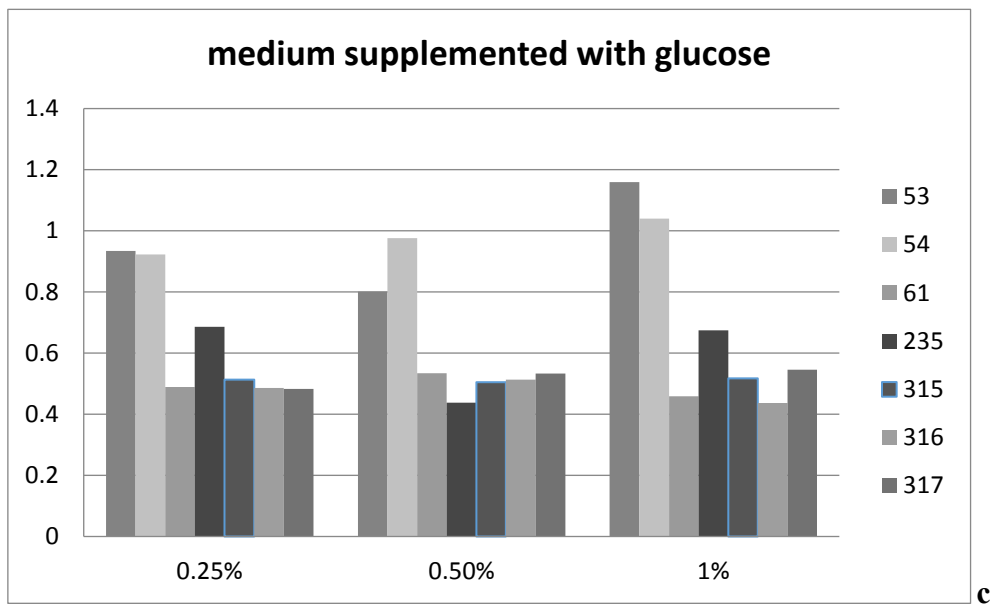


Figure 1 a-c Growth rates of *S. vacuolatus* strains grown under different conditions.
a. pH 3,5: 6.0 and 8.0; b. NaCl 0,25%-3%; c glucose 0.25% -1%

3.2 Effects of CO₂ concentration in the gas phase on growth and POX activity

The effects of CO₂ concentration in the gas phase were investigated in VBC. Fig. 2a and 2b reports the data obtained with two strains of *Scenedesmus vacuolatus*, 053 and 316 grown in VBC fed with only air gas stream.

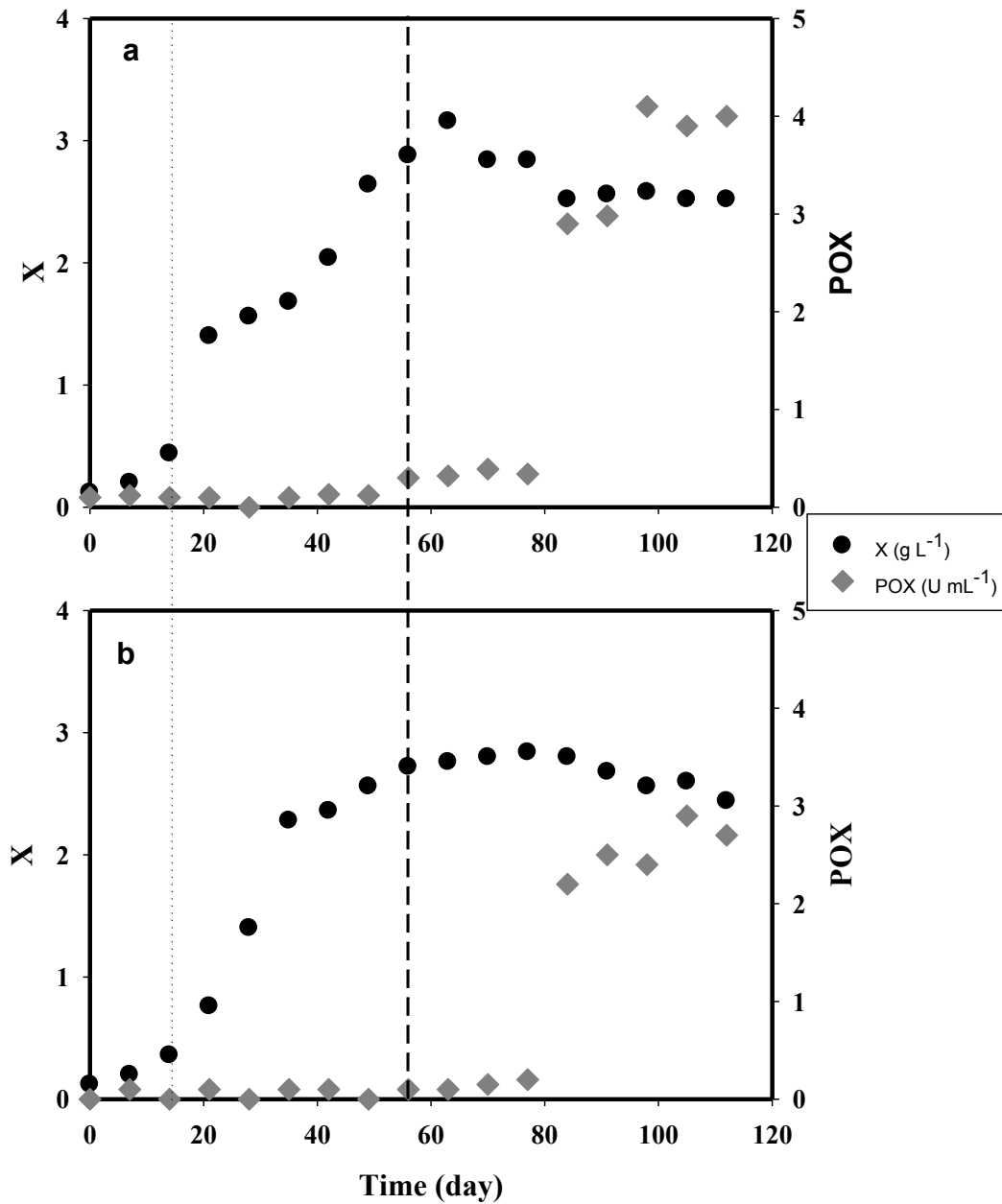


Figure 2: Semi-continuous cultures with only air to gas stream in VBC. (a) *S. vacuolatus* 053; (b) *S. vacuolatus* 316. Dotted line marks the start of fed-batch mode. Dashed line marks the start of semi-continuous mode.

The culture was carried out under batch conditions with respect to the liquid phase for 14 days: the algal biomass concentration increased up from 0.1 to 0.5 g L^{-1} for both strains. Fed-batch conditions started on day 14 and dotted vertical line marks the instant at which fed-batch mode started. Under fed-batch conditions microalgae growth constantly and the biomass concentration was, at $t=56$ day, about 3.16 and 2.84 g L^{-1} for *S. vacuolatus* 53 and 316, respectively; then the semi-continuous operation started. Dashed vertical line marks the start of weekly replacement of 35% of the suspension (dilution rate 0.05 d^{-1}).

Replacements were repeated for about 45 days. At t=100 day, microalgae cultivation was continued by supplementing BBM without nitrogen source for 10 days. Then the culture was stopped and the biomass was collected for the lipid analysis.

The results of the microalgae characterization under steady state conditions are reported in Table 2 for the tests carried out in VBC photobioreactors fed with only air to gas stream. Table 3 reported data obtained in VBC fed with 5% CO₂ supplemented air.

	<i>S. vacuolatus</i> strain 053 - 0.03%CO ₂	<i>S. vacuolatus</i> strain 316 - 0.03%CO ₂
X _{max} (g _X L ⁻¹)	3.16	2.84
X _{steady state} (g _X L ⁻¹)	2.77	2.73
P _X (g _X L ⁻¹ d ⁻¹)	0.138	0.136
A _X (g _X m ⁻² d ⁻¹)	1.385	1.365
Y _{X/E} (g _X E ⁻¹)	0.115	0.113
POX _{steady state} (U mL ⁻¹)	1.62	1.09

Table 2: Steady state data of semi-continuous tests in VBC photobioreactors with only air sparged.

	<i>S. vacuolatus</i> strain 053- 5% CO ₂	<i>S. vacuolatus</i> strain 316 - 5% CO ₂
X _{max} (g _X L ⁻¹)	6.4	5.98
X _{steady state} (g _X L ⁻¹)	4.85	4.67
P _X (g _X L ⁻¹ d ⁻¹)	0.243	0.233
A _X (g _X m ⁻² d ⁻¹)	2.426	2.335
Y _{X/E} (g _X E ⁻¹)	0.201	0.194
POX _{steady state} (U mL ⁻¹)	0.21	0.19

Table 3: Steady state data of semi-continuous tests in VBC photobioreactors with 5% CO₂ added to air .

Both strains of *Scenedesmus vacuolatus*, 053 and 316 were able to grow with or without CO₂ supplemented to air. The cultures aerated with air only never achieved the same biomass concentration, as also indicated in table 2 and 3. The biomass concentration achieved the highest value (4.85 and 4.67 g L⁻¹ for *S. vacuolatus* 53 and 316, respectively) in a photobioreactor with additional CO₂ concentration of 5%. The specific biomass productivity (P_X) and areal productivity (A_X) was about 0.243 and 0.233 g L⁻¹ d⁻¹ and 2.43 and 2.33 g m⁻² d⁻¹ for *S. vacuolatus* 95 and 316, respectively. The photon yield, in the

culture with 5% CO₂ supplemented to air, was about 0.201 and 0.194 g E⁻¹ for *S. vacuolatus* 53 and 316, respectively.

The results of phenoloxidase activity (POX) of *S. vacuolatus* 053 and 316 in the course of the experiments carried out in batch, fed-batch and semi-continuous conditions, indicate that both strains showed phenoloxidase activity only during the semi-continuous phase of growth, and only when no additional CO₂ was sparged in the photobioreactors,

3.3 Effects of CO₂ concentration in the gas phase on lipid content and FAME composition

The lipid content and FAME composition were also investigated for *S. vacuolatus* 053 and 316 grown in VBC with different CO₂ concentration in the gas phase. Table 4 report the data obtained with both strain grown in VBC with only air sparged (0.03% CO₂), while Table 5 data from *S. vacuolatus* strains grown with 5% CO₂ added to air.

	<i>S. vacuolatus</i> strain 053- 0.03% CO ₂		<i>S. vacuolatus</i> strain 316 - 0.03% CO ₂	
	N+	N-	N+	N-
%Lipid (g _{Lipid} gX ⁻¹)	13.37	15.38	12.31	14.59
%Linolenate (g g _L ⁻¹)	1.03	2.74	2.57	2.16
%Oleate (g g _L ⁻¹)	21.36	30.58	14.95	12.48
%FAME (g _{FAME} g _L ⁻¹)	22.39	33.32	17.52	16.64

Table 4: lipid content and FAME composition of *S. vacuolatus* 053 and 316 cultivated in VBC with only air sparged. N+ indicates that the culture is not in nitrogen starvation, while N- indicates that the culture is in nitrogen starvation.

	<i>S. vacuolatus</i> strain 053 - 5% CO ₂		<i>S. vacuolatus</i> strain 316 - 5% CO ₂	
	N+	N-	N+	N-
%Lipid (g _{Lipid} g _X ⁻¹)	29.84	24.83	21.06	23.92
%Linolenate (g g _L ⁻¹)	4.41	4.72	4.97	3.87
%Linoleate (g g _L ⁻¹)	1.34	0.70	0.50	0.98
%Oleate (g g _L ⁻¹)	18.61	25.93	22.66	19.13
%FAME (g _{FAME} g _L ⁻¹)	24.36	31.35	28.13	23.98

Table 5: lipid content and FAME composition of *S. vacuolatus* 053 and 316 cultivated in VBC with 5% CO₂ added to air. N+ indicates that the culture is not in nitrogen starvation, while N- indicates that the culture is in nitrogen starvation

The total extracted lipids (%Lipid) are the percentage of lipids within algal cells which were extracted successfully. The total lipid fraction achieved their maximum at 5% additional CO₂ sparging with *S. vacuolatus* 95 with values of 29.84% in nitrogen sufficient condition. The values in N starvation, in all condition and for both strain, were similar. A definitely lower value was obtained with air sparged (0.03% CO₂) for both strain.

The total esterified lipids (%FAME) are the percentage of total lipids within algal cells which were esterified successfully. It is defined as the amount of lipids which can be used as biodiesel. The highest amount of FAME was achieved with *S. vacuolatus* 053 grown in VBC with only air sparged (33.32%) during nitrogen starvation, but similar result was obtained for *S. vacuolatus* 053 grown in VBC with 5% CO₂ added to air (31.35%).

Table 4 and 5 reported the differences of lipid compositions under nitrogen sufficient conditions and nitrogen starvation conditions, too. The amount of Linolenate was the highest (with about 4.97%) in the cultures grown at 5% CO₂ with *S. vacuolatus* 316 in N sufficient condition. However, Linolenate showed similar results for both strain in different condition. Linoleate never appeared in tests carried out with only air sparged, while in cultures with CO₂ sparged, it was about 1% for both strain. Oleate was the dominating fatty acid in all cultures with a maximum at 0.03% CO₂ sparging under nitrogen starvation

(30.58%). The culture of *S. vacuolatus* 316 grown with only air sparged showed the lowest Oleate content (12.48%).

5. Conclusions

After the screening of the strains, *Scenedesmus vacuolatus* 053 and 316 result the two best candidates for experiments.

When the two strains were tested, addition of 5% CO₂ positively influenced lipids productivity and doubled the biomass values.

Between the two strains, *S. vacuolatus* 053 overall appears to be a good source for lipid productivity and biomass yield: in presence of CO₂ addition it achieved a biomass yield around 6.4 g L⁻¹ and a lipid contents around 29.84% of the total. These results are certainly competitive with data obtained from other *Scenedesmus* strains: *S. obliquus* in presence of 5% CO₂ achieved a maximum biomass values around 2.4 g L⁻¹ and a lipids productivity around 44,5% (Sforza et al. 2014) and in presence of 2,5% a biomass yield of 2, 1 g L⁻¹ and lipids contents of 10% (Shi et al. 2012); eventually *S. quadricauda* achieved a biomass value around 1,651 1 g L⁻¹ and 33% lipids contents (Dev Goswaimi et al. 2011).

Also in the case of FAME productions, the yield is higher in the strain 053 and achieved values around 30% of the contents. However, the production of FAME had different compartments in the two microorganisms: indeed in the strain 053 the FAME yield was influenced by the N starvation but not from the CO₂ addition while the strain 316 had the opposite behaviour. This fact probably is due to a different metabolism of the organisms.

S. vacuolatus 053 shown also a better POX activity than the strain 316 but only in presence of air sparged. Indeed this activity was completely suppressed by presence of 5% CO₂ in the gas phase although this suppression did not affect algae growth or lipids biosynthesis.

The obtained data induce to focus on the strain *Scenedesmus vacuolatus* 053. Already some works are in progress to evaluate the photosynthetic response and the attachment capacity of this organism (Carbone et al. 2016; Gargano et al. 2015a; Gargano et al. 2016b). These type of results could help to optimize the productivity of *S. vacuolatus*.

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CHAPTER THREE

Kinetics of photochemical steps in microalgae under different cultivation strategy

AUTHORS

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Abstract

Objectives Estimate the light intensity which maximizes the photosynthesis in microalgal cultures by means of a kinetic model of the photochemical process.

Results The photosynthetic performances of *Scenedesmus vacuolatus* under batch, fed-batch, semi-continuous cultivation strategy and with only air sparged or CO₂ added to air were quantified by means of gas exchange and pulse amplitude modulated fluorimetry (PAM). The kinetics of the photochemical processes were determined processing data from PAM using a model based on the state of the photosynthetic units. The photosynthesis rate resulted one order of magnitude slower in the cultures grown with only air with respect to those in which CO₂ was added to air. Both PAM and gas exchange techniques allowed to identify similar values of light intensity to maximize the photosynthesis rate under all the cultivation strategies.

Conclusions New tubular photobioreactor was proposed to avoid the photochemical process inhibition.

Keywords: microalgae, photosynthesis, *Scenedesmus vacuolatus*, photobioreactor, pulse amplitude modulated fluorimetry

Introduction

Light is one of the most critical factors on intensive microalgal cultivation (Olivieri et al., 2014). Indeed, light should be provided at the appropriate intensity and wavelength. Low light levels can be growth-limiting, while excessive light intensity may photoinhibit the photosynthetic process, and therefore a decrease of the growth rate (Chen et al., 2008, Carvahlo et al., 2006). Several kinetic models have been developed to describe this behavior in microalgae: Eilers and Peeters (1998) proposed a model of photosynthetic reaction centres based on relationship between light intensity and rate of photosynthesis. Modification of this model considered also photoadaptation, photoinhibition and flashing light effect (Bannister, 1979; Bernardi et al., 2014; Camacho Rubio et al., 2003; Garcia-Camacho et al., 2012; Han et al., 2000; Nikolaou et al., 2015).

Eilers and Peeters considered three possible state for the reaction center: open or resting state (1) in the dark, closed or activated state (2) in the presence of light, damaged or photoinhibited state (3) due to excess of light (figure 2).

x_1 , x_2 , x_3 denote the fractions of the reaction centers respectively in the states 1, 2 and 3. The transition through the states is represented by four kinetics parameters: α , β , γ , δ .

These parameters characterized four itineraries of the reaction centres:

1) The open state (1) is activated by the photon captures and jumps to the close-state (2).

The photon capture rate is characterized by light intensity (I), x_1 and the kinetic parameter α :

$$r_{PC} = \alpha I x_1$$

2) The close-state (2) returns to the open state (1) thanks the photochemical quenching. The rate of photochemical quenching is described by γ and x_2 .

$$r_{PQ} = \gamma x_2$$

3) In presence of excess amounts of photons the close state (2) turns into photoinhibited state (3) and releases heat. The rate of the photoinhibition is associated with light intensity (I), x_2 and β .

$$r_{PI} = \beta I x_2$$

4) The photoinhibited state (3) doesn't participate to photosynthesis process but recovers the open state (1) by repairing process.

The rate of this process is characterized by δ and x_3 .

$$r_{REP} = \delta x_3$$

Recently, Gargano et al.,(2015) proposed a simple procedure to assess the kinetic parameters by pulse amplitude modulation fluorimetry . The suggested procedure, applied on several microalgal strains and different photobioreactor designs, indicated the possibility, once the kinetic parameters assessed, to identify the irradiance range under which the photochemical process is controlled by the photons capture and the photoinhibition competes with the photochemical quenching. Moreover, the results pointed out that the photosynthetic kinetic parameters are specific for each microalgal strain and depend on the adaptation to light irradiance (Gargano et al., 2015).

The aim of the present paper is to investigate the effect of cultivation strategy (batch, fed-batch and semi-continuous) and the influence of CO₂ addition in the gas phase on the dynamic of photochemical steps involved in the Eilers and Peters' model. The results of the efficiency of the photochemical process were integrated with the gas exchange analysis. With this approach, the optimal values are assessed of light intensity that maximize the photosynthesis rate under all the cultivation strategies.

Scenedesmus vacuolatus was chosen because it is considered one of the best candidate for biodiesel production (Mata et al., 2010; Schenk et al., 2008) and for phycoremediation. (El-Sayed and Maguid, 2010; Pollio et al., 1993).

Materials and Methods

Microalgal strains and medium

Scenedesmus vacuolatus 053/95 strain was used. The strain was obtained from the Algal Collection of University Federico II in Naples "ACUF" (<http://www.biologiavegetale.unina.it/acuf.html>). Modified Bold Basal Medium (BBM) as reported by Olivieri et al. (2013) was used. The medium was autoclaved for 20 minutes at 120 °C. The final pH of the medium was close to 7.

Photobioreactor design and operating conditions

Microalgae were growth in vertical bubble column photobioreactors. Design details can be found elsewhere (Olivieri et al., 2012). The irradiance level was set at 250 $\mu\text{E}/(\text{m}^2 \text{s}^{-1})$. The CO₂ concentration in the gas stream fed to the photobioreactors was increased from only air sparged to 2%v/v. Tests were carried out under batch conditions for about one week. The shift to the fed-batch phase took place when the nitrogen concentration in the medium achieved a value lower than 10 mg L⁻¹. Under fed-batch mode, ten times

concentrated medium was added to the cultures twice a week in order to avoid the nutrient starvation and to allow the continuous cell growth. The final volume of the culture was kept constant because of the water withdraw due to the samples and to the water stripping by gas bubbles. The shift to the semi-continuous phase took place when the biomass concentration achieved a value close to 3.5 g/L. Under the semi-continuous mode, 30% of culture was replaced with three times concentrated fresh medium every seven days in order to achieve a dilution rate equal to 0.043 d⁻¹ and to replace the initial concentration of the nutrient. The biomass and medium collected under batch, fed-batch, semi-continuous was characterized. Microalgal cultures were carried out in duplicate for each set of operating conditions.

Medium characterization

pH: the pH was measured with a pH meter (benchtop pH meter Mettler Toledo).

Nitrate: the analysis of nitrate concentration in the medium was made using a nitrate electrode (Mettler-Toledo).

Biomass characterization

Biomass concentration: the biomass concentration was estimated by measuring the optical density at 600 nm with a spectrophotometer (Specord 50 – Analytic Jena). The dry weight of the inoculum was used to also established.

Chlorophyll a content: the in vivo chlorophyll a content was measured by a fluorometer (AquaFluorTM; Handheld Fluorometer/Turbidimeter; Turner Designs).

Gas exchanges

Oxygen evolution in the samples was measured in a oxygraph (Oxygraph Hansatech) equipped with a thermostated control set at 24 °C and with S1 Clark-type oxygen electrodes. Different light intensities (PFR) were supplied ranging from 43 to 600 PAR. The oxygen control unit allowed to observe the oxygen consumption during the O₂ uptake under the dark phase (respiration, R_d) and the oxygen production under the light phase (photosynthesis, P_I). The real photosynthetic rate (P), expressed as μmol_{O2} μgChl_a⁻¹ s⁻¹, was calculated by means of the following equation (Mercado et al., 2004):

$$P = P_{max} \left[\frac{\alpha_l PFR}{(P_{max} + \alpha_l PFR)} \right] + R_d \quad [1]$$

Where the maximal photosynthetic rates (P_{max}) and the ascending slope at limiting PFRs (α_l) were obtained from the fit of the curves to the equation provided by Henley (1993). The onset of saturation light (E_k) was measured as proposed by Mercado et al., (2004). It resulted to be:

$$= E_k = \frac{P_{max}}{\alpha_l}$$

[2]

The biomass concentration was always reported to 0.12 g/L for all gas exchange measurements in order to avoid the light limitation. The values of oxygen measured in dark and light condition were normalized for *Chlorophyll a* content in the culture.

Gas exchange measurements were carried out in triplicate for each set of operating conditions.

Fluorescence measurements

Chlorophyll fluorescence emissions were determined at room temperature by a pulse amplitude modulated fluorimetry (Hansatech Fluorescence Monitoring System) elsewhere schematized (Gargano et al., 2015). Microalgae, before the measurements started, were adapted to darkness for 30 minutes; during all experiments, the sample was continuously stirred. The analysis were based on two different measurement techniques, chlorophyll response to light pulse (LP) and saturating light pulse under modulated light (ML) conditions and chlorophyll response to saturating light pulse (SP) under actinic light conditions (AL).

Fluorescence measurements were carried out in triplicate for each set of operating conditions.

Assesment of kinetic parameters through the model of Gargano et al.

The kinetic parameter were assessed by processing fluorescence data with a code developed in Matlab programming language.

α and γ were estimated through the elaboration of fluorescence data achieved with light pulse (LP) under (ML) condition.

Indeed in these conditions pulse actives the closed state but not the photoinhibited state and therefore only the transition of the population of reaction centers from x_1 to x_2 or x_2 to x_1 is possible.

β was estimated through the elaboration of fluorescence data realised with saturating pulse (SP) under ML condition.

During this pulse there is a decay of the fluorescence and consequently only the transition from state 2 to state 3 is possible.

δ was estimated throught the elaboration of fluorescence data realised during saturating pulse (SP) under Actinic light (AL) conditions.

Indeed fluorescence in this condition is due to the transition among the three states.

Results and Discussion

Biomass growth

Figures 1 shows data for cultures of *Scenedesmus vacuolatus* carried out in vertical bubble column photobioreactor feeding with air supplemented with CO₂ at 2.0 %.

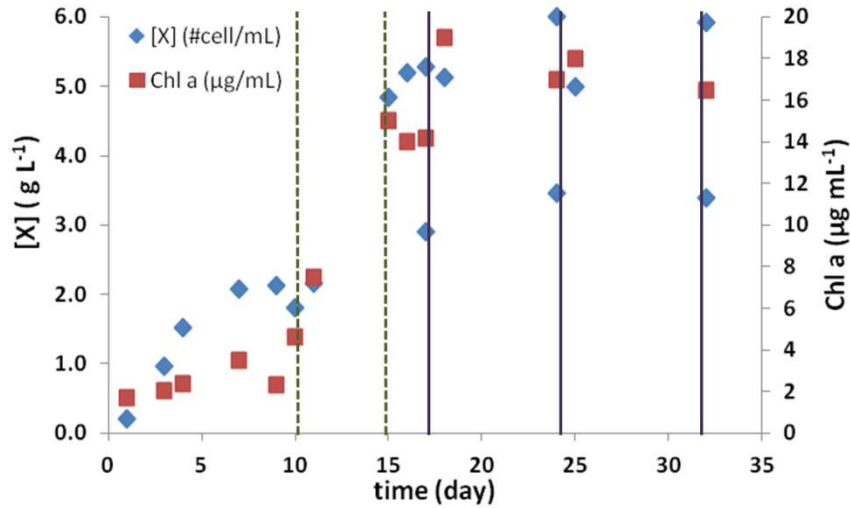


Figure 1: Cultures of *S. vacuolatus* at varying the operating conditions. Dotted lines mark BBM addition under fed-batch mode. Continuous lines mark culture replacement under semi-continuous mode. (◆) the biomass concentration $[X]$; (■) the Chlorophyll *a* content (Chl *a*).

The biomass concentration increased up to 2.0 g/L in the batch phase and then achieved a maximum value of about 5.5 g/L at the end of the fed-batch phase. The semi-continuous mode started after nineteen days and it was characterized by a weekly replacement of 30% of the suspension with a three times concentrated medium. The achieved dilution rate was 0.043 d^{-1} . Provided that a steady state condition was established, the reactors were operated under semi-continuous mode for about three weeks. The biomass collected during the forty days of cultivation - from batch, fed-batch, and semi-continuous mode - was analyzed on line for the fluorescence analysis and gas exchange analysis.

The *Chlorophyll a* content was about 0.5 µg/mL at the beginning of the tests and increased with the time in the cultures due to biomass increase. As a consequence of the CO_2 addition, the average pH in the cultures was constant at about 7 (data not shown).

A similar trend was obtained for *S. vacuolatus* cultures grown with only air sparged (data not shown). The cultures were carried out under batch condition for one week. When the nitrate concentration in medium became lower than 10 mg/mL, the fed-batch mode was started. The biomass concentration at the end of batch phase resulted be 1.44 g/L while the *Chlorophyll a* content 1.3 µg/mL. The fed-batch phase was conducted for ten days for which the biomass concentration achieved 2.16 g/L whereas *Chlorophyll a* content was 8.4 µg/mL. At the end of the fed-batch phase, the semi-continuous mode started. A semi-continuous phase, characterized by a dilution rate equal to 0.043 d^{-1} , was conducted for three weeks until the biomass steady state was achieved. The biomass concentration at steady state was 1.6 g/L while the *Chlorophyll a* content reached a value of 3.8 µg/mL.

Photosynthetic behavior by fluorimetric analysis

The procedure to assess the kinetic parameters of the photochemical processes reported by Gargano et al.(2015) was used with reference to samples of *Scenedesmus vacuolatus* collected during batch, fed-batch and semi-continuous operating condition. The α and γ kinetic parameters, reported in figure 2, were calculated on biomass in dark adapted condition using pulse characterized by a light intensity ranging from 500 to 3000 $\mu\text{E}/(\text{m}^2 \text{s}^{-1})$ and a pulse amplitude from 0.6 to 2 sand pulse amplitude 0.6 s. The β constant was estimated on biomass in dark adapted condition using saturating pulse characterized by light intensity ranged from 3000 $\mu\text{E}/(\text{m}^2 \text{s}^{-1})$ and pulse amplitude from 0.8 to 2 s to 4000 $\mu\text{E}/(\text{m}^2 \text{s}^{-1})$ and pulse amplitude 0.8 . The δ term was calculated on biomass in light adapted condition, light condition ranged from 40 to 1350 $\mu\text{E}/(\text{m}^2 \text{s}^{-1})$ while saturating pulse was set at 3000 $\mu\text{E}/(\text{m}^2 \text{s}^{-1})$ for 0.6 s.

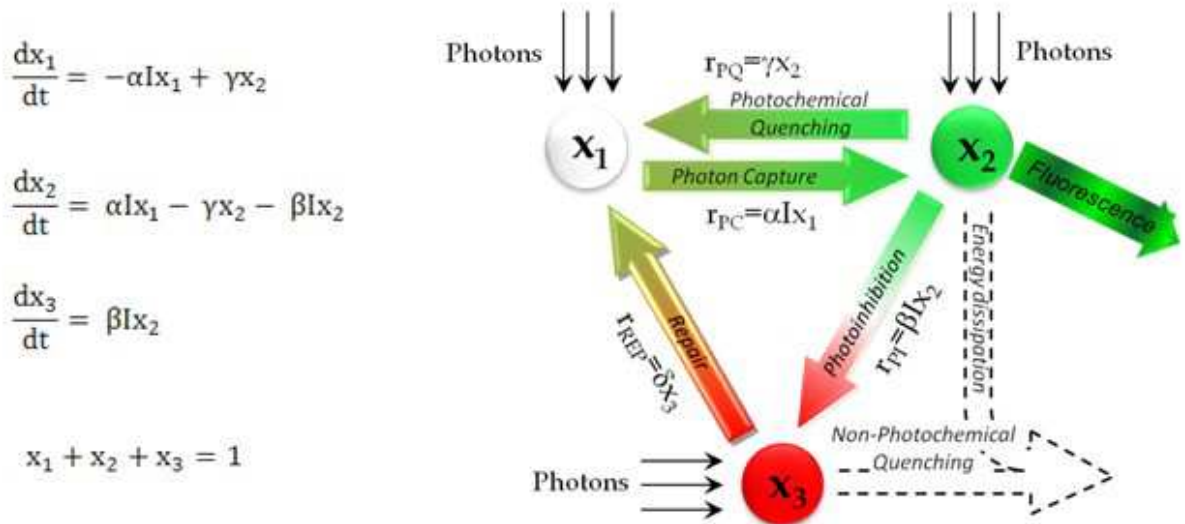


Figure 2: Schematic representation of the kinetic model proposed (from Gargano et al., 2015).

Table 1 reports the values of kinetic parameters of the photochemical processes assessed for *S. vacuolatus* cultures carried out in vertical cylindrical bubble column photobioreactors under batch, fed-batch and semi-continuous mode.

	BATCH	FED-BATCH	SEMI-CONTINUOUS
α (m ² /μE)	1.55e-3±5.7e-4	1.13e-3±8.47e-4	9.51e-4±1.81e-4
β (m ² /μE)	2.78e-4±0.54e-4	5.56e-4±6.67e-5	2.58e-4±5.80e-5
γ (s ⁻¹)	5.3±0.90	3.99±0.54	4.18±0.19
δ (s ⁻¹)	1.02e-2± 0.32e-2	2.39e-2±0.17e-2	8.77e-3±1.90e-3

Table 1: Kinetic parameters of the photochemical processes assessed for *S. vacuolatus* cultures under batch, fed-batch and semi-continuous operating condition with air supplemented with CO₂ at 2.0 %.

The analysis of the data reported in table 1 points out that the operating conditions of microalgal cultures seems to affect the fluorescence kinetic parameters. Indeed the value of β and δ are significantly larger in case of fed-batch operation.

Instead the time-scale of the reaction photon capture and discharge rate (α and γ) rate were not influenced by the operating condition and biomass concentration. The comparison of the time scale of the four reactions along the operating condition and biomass concentration supports the identification of the bottleneck for the photochemical process. The rate of photochemical quenching and repair process were not influenced by operating condition and the time scale of the repairing process were always longer than the other time scales for almost all irradiance allowed in natural environmental (2000 – 3000 μE/(m² s)). As reported by Gargano et al. (2015) the repairing process represents the bottleneck of the photochemical process when the operating conditions promote the formation of the inhibited-state.

The same protocol was also used to assess the kinetic parameters of the photochemical processes for the *S. vacuolatus* cultures grown with just air sparged. Results are reported in table 2.

	BATCH	FED-BATCH	SEMI-CONTINUOUS
α (m ² /μE)	2.34e-3±2.54e-4	4.02e-3±6.87e-4	1.27e-3±2.99e-4
β (m ² /μE)	1.85e-3±1.97e-4	7.87e-5±2.67e-5	1.17e-4±1.98e-5
γ (s ⁻¹)	5.99±0.41	6.83±0.39	6.75±0.26
δ (s ⁻¹)	1.73e-3±2.47e-4	1.92e-3±3.65e-4	8.31e-4±1.11e-4

Table 2: Kinetic parameters of the photochemical processes assessed for *S. vacuolatus* cultures under batch, fed-batch and semi-continuous operating condition with justair sparged.

The results confirmed that biomass adapts to operating conditions and the photochemical process depends on the photobioreactor design and operating conditions (Gargano et al., 2015). At biomass concentration higher than 0.5 g/L, the photochemical process was not influenced by the biomass concentration, confirming that biomass concentration higher than 1 OD reduces the total amount of light to zero in 1 cm. The photon capture resulted always slower in the cultures growth with only air added comparing with the cultures growth with 2% CO₂ added to air. No real differences were observed on the time scale that characterize the photoinhibition and the photochemical quenching. The repairing process resulted faster in the cultures growth with CO₂ added to air, but it was still the limiting step of the photochemical process when some reaction centres are in the x₃ state. As reported by Serôdio et al. (2009), the fluorescence was influenced by the physiological status of the microalgae.

Gargano et al. (2015) proposed the equation 3 for x₂ in steady state conditions; starting from it, it is possible to calculate the light intensity that maximize the growth rate. Considering the conclusion of the paper of Gargano et al., 2015, it should be optimized the time in which the biomass is under light condition and under shadow condition.

$$= x_{2,ss} = \frac{\alpha \delta I}{\gamma \delta + (\alpha + \beta) \delta I + \alpha \beta I^2} \quad [3]$$

The light intensity (I_{opt}) that maximizes the photochemical process avoiding the inhibited state (x₃) can be calculated from model (3):

$$= I_{opt} = \sqrt{\frac{\delta \gamma}{\alpha \beta}}$$

and results are reported in table 3.

		BATCH	FED-BATCH	SEMI-CONTINUOUS
2% CO ₂	$I_{opt} (\mu\text{E}/(\text{m}^2 \text{s}^1))$	354	390	386
only air	$I_{opt} (\mu\text{E}/(\text{m}^2 \text{s}^1))$	154	204	161

Table 3: Calculated value of the light intensity (I_{opt}) that maximizes the photochemical process.

The value of light intensity (I_{opt}) that should avoid the formation of x_3 resulted to be quite constant at varying the operating conditions for the same cultures conditions, but higher in the cultures with CO₂ added to air than with only air sparged. The CO₂ limitation influenced the photochemical process.

Photosynthetic behavior by gas exchange

The photosynthetic process was analyzed by means of gas exchange measurements. The biomass was always fixed at a 0.12 g/L. The light intensity was increased from 0 to 600 $\mu\text{E}/(\text{m}^2 \text{s}^1)$. Typical photosynthesis/respiration (P/I) curves obtained for both operating conditions and different operating mode are reported in figure 3. The *Chorophyll a* content expressed $\mu\text{g}/\text{mL}$ was utilized to normalize the data of oxygen evolution. Data were used to estimate the value of the parameters characteristic of the photosynthesis: the initial slop of the P/I curve (α_i) and the saturating light irradiance value (P_{\max}) according to Eq. (1).

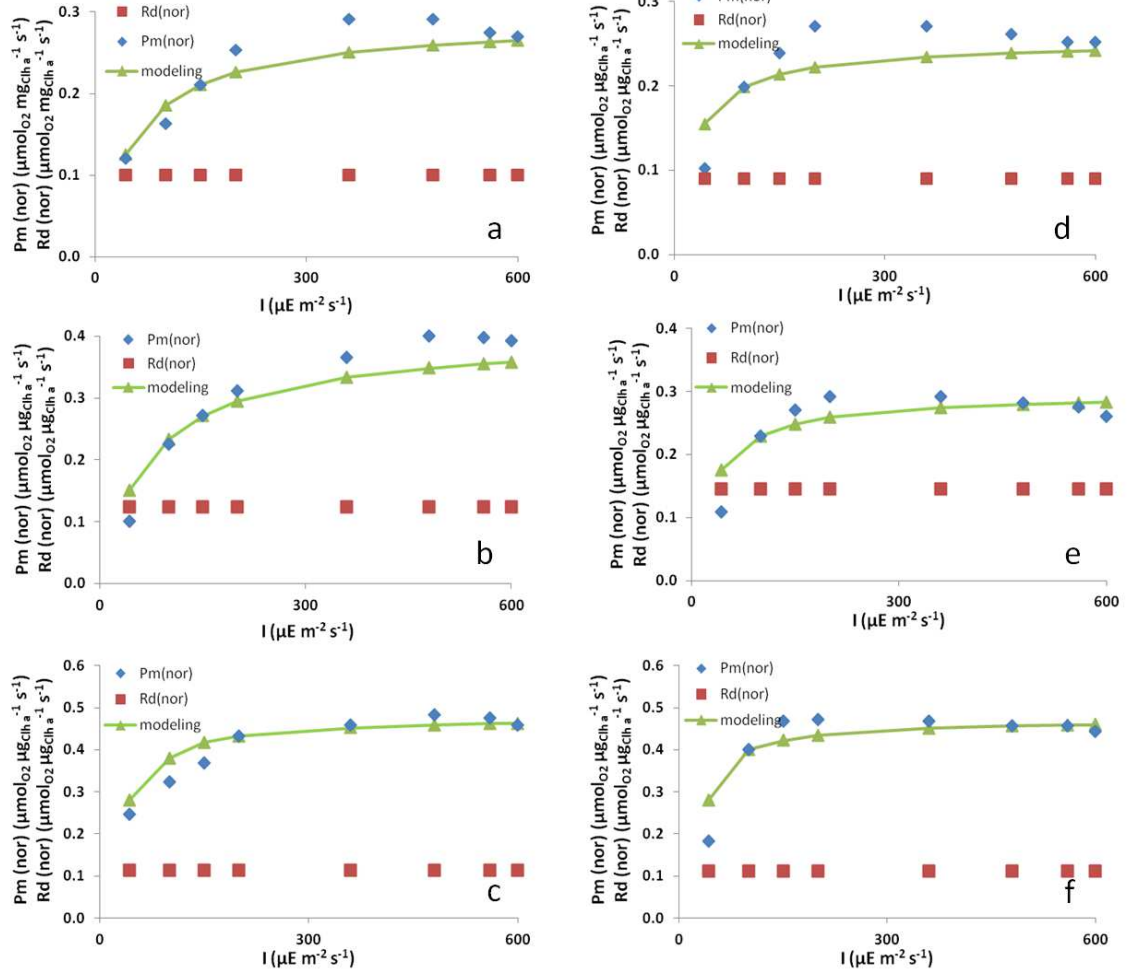


Figure 3: Photosynthesis and respiration curve (P/I curve) of *S. vacuolatus* cultures growth under

different operating conditions. The red squares represents the respiration in the dark ($R_{d(nor)}$), the blue rhombus the photosynthesis activity ($P_{m(nor)}$), the green triangles the modeling results (modeling). a) culture with CO_2 added to air in batch phase; b) culture with CO_2 added to air in fed-batch phase; c) culture with CO_2 added to air in semi-continuous phase; d) culture with only air sparged in batch phase; e) culture with only air sparged in fed-batch phase; f) culture with only air sparged in semi-continuous phase.

The best fitting values for α_I and P_{max} are reported in table 4. The onset of light saturation (E_k), calculated by using Eq. (2), is also reported .

		P_{\max} [$\mu\text{molO}_2/(\mu\text{gChl a s})$]	α_I [$\mu\text{molO}_2/(\text{mgChl a s/}$ $(\mu\text{E/m}^2 \text{ s}))$]	E_k [$\mu\text{E/m}^2 \text{ s}$]
batch	with CO ₂ added	0.29	0.0009	322
	with only air sparged	0.27	0.0011	226
fed-batch	with CO ₂ added	0.40	0.0012	333
	with only air sparged	0.30	0.0012	239
semi-continuous	with CO ₂ added	0.48	0.0012	400
	with only air sparged	0.46	0.0018	255

Table 4: Parameter of photosynthesis (P_{\max} and α_I) and onset of light saturation (E_k) for all operating condition analyzed.

The highest rate of photosynthesis at saturating irradiance (P_{\max}) was obtained in semi-continuous operating mode, while the lower value in batch condition when the biomass concentration was lower. The initial slope of the P/I curves (α_I) did not change with the operating conditions and it ranged from 0.0009 to 0.0018 $\mu\text{molO}_2/(\text{mgChl a s})$ ($\mu\text{E}/(\text{m}^2 \text{ s}^1)$). As reported by Mercado et al., 2004, the α_I parameter is specific for the microalgal species and it is not influenced by the operating conditions. The values of E_k varied with the operating conditions. In particular, E_k was higher in the cultures grown with 2% CO₂ added to air, and it ranged from 322 to 400 $\mu\text{E}/(\text{m}^2 \text{ s}^1)$, while it was lower in the cultures with only air sparged and maximum achieved value was 255 $\mu\text{E}/(\text{m}^2 \text{ s}^1)$. The effect of the CO₂ added to air in the cultures on P_{\max} and E_k showed a clear pattern. Thus, P_{\max} increased in the cultures with the increasing of biomass concentration and chlorophyll a content.

It is interesting to observe that the values of I_{opt} and E_k are quite close each other, despite the totally different principle behind the PAM and the photosynthesis rate measurements: the first one is mainly dependent by the wall irradiance of the light pulse, since also the fluorescence response is detected at the wall; on the other hand the photosynthesis rate is a volumetric measurement performed by the oxygen sensors located at the bottom of the sample.

It is also interesting to observe that in real photobioreactor the value of I_{opt} is achieved by the average level of irradiance within the system as reported by Olivieri et al., (2014, 2015) under well mixed conditions. This value is determined by not only the wall irradiance, but

also by the light path and cell density. Thick and diluted cultures have in principle similar average irradiance as thin and very concentrated cultures.

Photoinhibition prevented by photobioreactor design

The results, reported in table 3 for I_{opt} values and confirmed in table 4 by E_k values, suggest that there is a specific light intensity that maximizes the photochemical process avoiding the photoinhibition. The photoinhibition decreases the biomass productivity and high light intensity should be avoided. Therefore, a new photobioreactor should be suggested. Figure 4 represents an ideal photobioreactor that consider this specific light intensity.

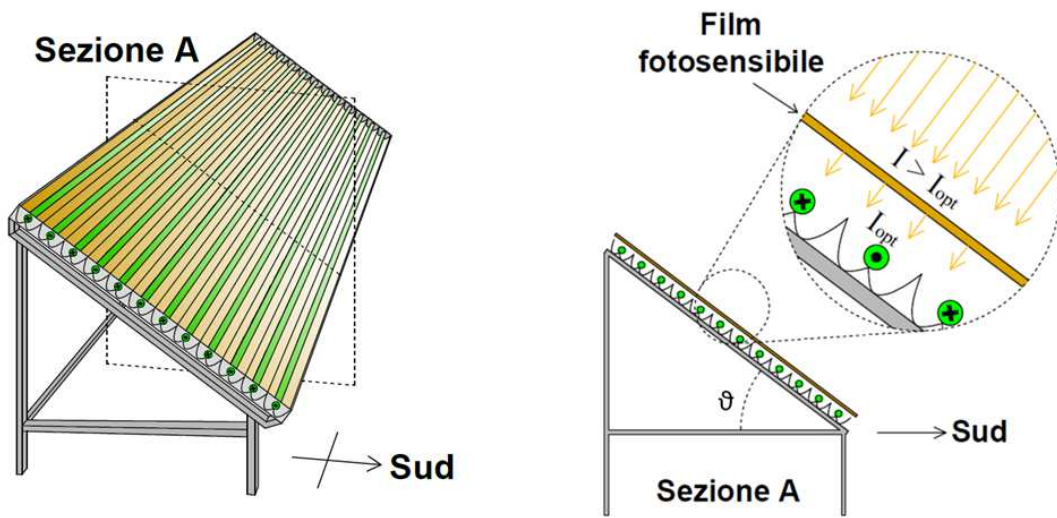


Figure 4: Sketch of the proposed tubular photobioreactor.

It is a solar collector photobioreactor CPC oriented from East to West and inclined with angle ϑ equal to the latitude where the reactor is placed. The photobioreactor is covered by a photochromic photosensitive panel able to reduce the light intensity at the fixed value. In this way the circadian clock, characteristic of microalgae, is preserved and the light is never so high to inhibit the photosynthetic process.

Conclusions

The kinetic parameters of the photochemical process were successfully estimated in all operating conditions using the simple procedure proposed by Gargano et al. (2015). The analysis of the data pointed out that the operating culture mode and biomass concentration did not affect the photosynthetic kinetic parameters. The photochemical process is not influenced by the operating mode, but it depends on the operating conditions. The photon capture resulted always slower in the cultures grown with just air sparged comparing with the cultures growth with air supplemented with CO_2 at 2%. No significant differences were

found in the photoinhibition and the photochemical quenching time scale. The repairing process resulted faster in the cultures growth with CO₂ added to air, but it still represents the bottleneck of the photochemical process when the operating conditions promote the formation of the inhibited-state. The light intensity (I_{opt}) that maximizes the photochemical process by avoiding the inhibited state (x_3) resulted quite constant varying the operating conditions, but it resulted higher in the cultures with 2% CO₂ added to air. Using the equation reported by Mercado et al., 2004, it was possible to calculate the onset of light saturation (E_k) by means of gas exchange measurements. The values of E_k varied with the operating conditions but not with the operating mode. In particular, E_k was higher in the cultures growth with 2% CO₂ added to air, and lower in the cultures with only air sparged. There is a strong correspondence in the value of light intensity, I_{opt} , calculated by the model adopted and E_k calculated using gas exchange measurements.

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CHAPTER FOUR

Evaluating microalgal attachment to surfaces: a first approach towards a laboratory integrated assessment.

Evaluating microalgae attachment to surfaces: a first approach towards a laboratory integrated assessment.

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Abstract

Recently, the challenge of producing algal biomass at low cost has been faced also with manufacturing systems based on immobilized microalgae. The growth of microalgae as a biofilm reduces the costs of harvesting and also the water demand, allowing at the same time a high biomass productivity. The initial adhesion to a surface is one of the key factors for the formation and maintenance of a stable microbial community, and although the physical properties of the surface have an influence on the adhesion process, the major role is played by species selection. Here we propose a simple procedure to establish on a bench-scale the ability of an algal strain to form a stable biofilm on a surface. As a model organism was selected *Scenedesmus vacuolatus* (ACUF 053) and the progressive adhesion of the microalgae at the surface of two tissues, cotton and jute, was followed with a two-step protocol based on the combination of image analyses and fluorometric measurements. The growth and viability of the algal biofilm were followed through color measurements of inoculated carriers taken at different times using the Trainable Weka Segmentation (a plugin of Fiji) on digital photographs, whereas pulse amplitude modulation (PAM) fluorometry allowed the measurement of algal photochemical activity on two textures. The results indicate that the progressive adhesion of the microalgae reached 80% of the surface of cotton fabrics during the first four days, and that the indicators of cell photosynthetic performance, decreased during the time course of the experiment, suggesting that the reduction of the nutrients concentration in the media could be responsible of progressive

decay of the photochemical activity. In conclusion, the method provides reliable data on the extent and metabolic efficiency of the algal attachment to a solid substrate.

Key words – microalgae, *Scenedesmus*, biofilm adhesion, image analysis, PAM fluorometry

1. Introduction

Mass cultures of microalgae are presently at a stage not fully developed, despite years of research. The reason for their reduced exploitation is the too high cost of production, and the efforts of the last years are particularly concentrated on this aspect. The challenge of producing algal biomass at lower costs has been faced also with manufacturing systems based on immobilized microalgae. The growth of microalgae as a biofilm reduces the costs of harvesting and also the water demand, allowing at the same time high biomass productivity (Liu et al., 2013). The initial adhesion of algae to a surface is one of the key factors for the formation of a biofilm, and is ruled by the properties of the surface and the species selection (Irving and Allen, 2011). The production of extracellular polymeric substance (EPS) by unicellular algae is known for decades (Hellebust, 1974), and a wide array of molecules, ranging from carbohydrates to proteins, lipids and vitamins has been detected in the external space that surrounds the cells (Myklestad, 1995), playing a key role in the establishment of the biofilm. The genus *Scenedesmus*, one of the first microalgae tested in suspended-based mass cultivation (Ketchum and Redfield, 1949), has shown a promising attitude to grow on the surface of solid carriers, and different methods of attachment have been proposed (Chen et al., 2014). Recently, we have concentrated our attention on the growth and photosynthetic performances of liquid cultures of *Scenedesmus vacuolatus* in enclosed photobioreactors. The description of photosynthesis behavior of *S. vacuolatus* through a kinetic model by PAM fluorometry was carried out with the aim of optimizing the biomass growth rate, and to assess the effects of different light operating conditions and photobioreactor design (Gargano et al., 2015). Then, the photosynthetic performances of *S. vacuolatus* under batch, fed-batch, semi-continuous cultivation strategy and under different CO₂ regimes were evaluated with the same model, to estimate the light intensity which maximizes the photosynthesis in liquid mass cultures. Here we propose a simple procedure to establish on a bench-scale the ability of an algal strain to form a stable biofilm on a surface. This two-step protocol is based on a combination of image analyses and fluorometric measurements, providing information on the extent and viability of

biofilm formation that can be very useful to assess the attitude of microalgal strains to grow on a solid substrate.

2. Materials and Methods

S. vacuolatus strain ACUF 053 (www.acuf.net) was maintained and grown on Bold's basal medium in 100 mL Erlenmeyer flasks placed in a climatic chamber at $24 \pm 2^\circ\text{C}$, on a shaking apparatus at 60 rpm. Continuous light, at $90 \pm 10 \mu\text{mol m}^{-2} \text{s}^{-1}$ was provided by 36 WT12 fluorescent Cool White (Osram light, Munich, Germany). The materials selected for adhesion experiments were cotton and jute, which were cut into square carriers 2,0 cm x 2,0 cm (width 0,3 cm) using a scissor. The carriers were first photographed with a Nikon 5300 digital camera and then, at higher magnifications, with a Leitz Metallurgical Microscope, equipped with a digital camera. Three carriers of each material type, previously sterilized at 120°C for 20 minutes, were placed in a 9 cm diameter glass Petri dish containing 30 mL of sterile BBM. Inocula of *S. vacuolatus* from exponential cultures were poured in each Petri dish, to have a final concentration of 0,5 optical density, assessed with a Specoman 50 spectrophotometer at 600 nm. The Petri dishes were placed on a rotating shaker at 70 rpm and held in the same conditions of light and temperature previously described for the maintenance cultures. Distilled water was added every two days to compensate the evaporation. The nitrogen concentration in the medium was measured as nitrate, by the spectrophotometric method reported by Collos et al. (1999). The adhesion of *S. vacuolatus* to the carriers was measured through a photographic recording of each carrier following the method described by Marasco et al. (2016). The carriers were placed on millimetric paper under controlled light provided by florescent lamps. The image were captured with a Nikon D5100. The parameters of the camera were: quality image FINE, image dimension NORMAL, length 3696 pixels, height 2448 pixels, horizontal resolution 300 dpi, vertical resolution 300 dpi, Bit depth 24, focus 5,6, exposition time 1/8, ISO sensibility 320, focal distance 50mm, light source AUTO. The digital images recorded at different times were analyzed with the software Fiji (Schindelin et al., 2012; and also <http://www.fiji.sc>), an open source image processing package based on ImageJ™ and the geometry and radiometry of the images were rectified to allow comparisons. The result of these geometric corrections is a multi-layer file, in which each layer represents a single measurement. The color measurement was analyzed using the Trainable Weka Segmentation (Hall et al., 2009) a plugin of Fiji, that permits to cluster different colours and their tones, distinguishing in this way the substrate from by

microalgae. Chlorophyll fluorescence emissions were determined at room temperature by a pulse amplitude modulated fluorometer (Hansatech Fluorescence Monitoring System) elsewhere schematized (Gargano et al., 2015). The Petri dishes were kept in the dark for 30 minutes before starting the tests. Since it is known that the humidity content of carriers is the key factor ruling fluorescence measurements in the lab, each carrier was moisturized for two times at an interval of ten minutes, according the procedure described by Eggert et al. (2006). The protocol used for the PAM analysis was reported by Maxwell and Johnson (2000), with some modification for photosynthetic and non-photosynthetic quenching times (3 minutes). The algae were exposed at three light intensity ($39\text{-}150\text{-}400\ \mu\text{mol m}^{-2}\text{s}^{-1}$). F_v/F_m were determined with a completely saturating white light pulse ($2600\ \mu\text{mol photons m}^{-2}\text{s}^{-1}$ weak 0,6). The gain of the instrument was 30 and the weak was 1. The light conductor of the fluorometer was always blocked at a distance of 7 mm from the substrate thanks to a special support. The fixed distance was necessary to avoid changes during fluorescence measurement. The measurements were carried out in triplicate for each set of operating strategies and conditions. The experiments were repeated for three times and the results were analyzed with ANOVA test.

3. Results

Cotton and jute fabrics were cut in small squares of 2 cm and were used as carriers for the experiments of *S. vacuolatus* cell immobilization. The experiments lasted 16 days, and the adhesion of the alga to substrates was followed by image analyses and PAM fluorometry. After 2 days, the colonization of the carriers by *S. vacuolatus* attained more than 80% of the surface of each carrier (not shown). Digital image analysis is a low-cost technique that allows the non-destructive recording and quantification of different components of a biofilm (Kaur and Kaur, 2014). Thanks to the colour cluster analysis (Trainable Weka Segmentation), it was possible to obtain from each image different ranges of hues and colours, which can be observed in terms of visible absorption spectrum. On both cotton and jute, the prevalent colour of the images taken before the inoculation and after the first 24 hours was white, that indicates the absence of algae on the carriers. After 48 hours and until the end of the experiment, the yellow colour prevailed, showing the formation of a subtle layer of microalga, which covered more than 80% of the total surface of both carriers. Finally, the green color occurs in the presence of large biomass of microalgae, indicating the formation of a thick biofilm (Berner et al., 2014), that accomplished to less

than 20% of the superficial coverage of both carriers at the end of the experiment (Figure 1a, b).

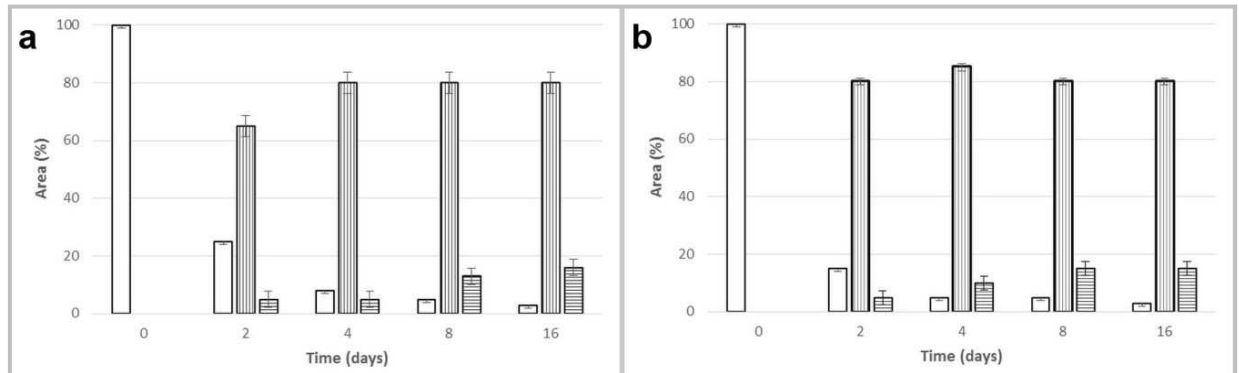


Figure 1. Growth of microalgae on cotton (a) and jute (b) carriers measured with the Trainable Weka Segmentation. Uncolonized area (□), colonized area (▨), biofilm (▤).

The nitrate content of the culture medium in which the carriers were immersed was progressively reduced to less than one tenth of the initial concentration (Figure 2). The depletion of nitrate is generally linked to an increase of the pH (Markau et al., 2014). In this experiment, the initial pH of the medium was 6,5 and achieved values around 9 at the end of the test. The nitrate depletion in the medium in the presence of cotton carriers was initially slower, but the final measurements evidenced similar values of nitrate concentration (4 mg/l) in presence of both carriers (not shown).

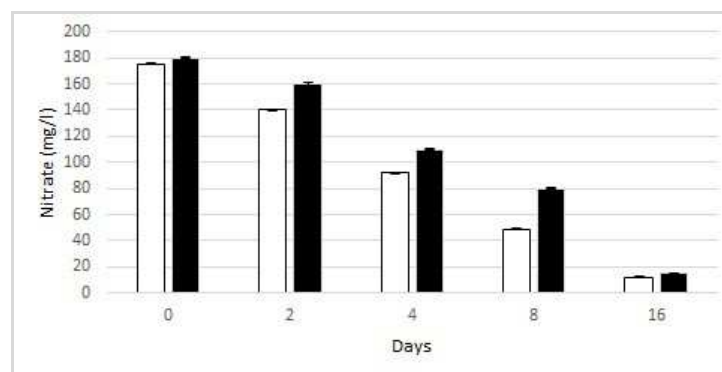


Figure 2. Nitrate content in the culture medium supporting the growth of the carriers. Cotton (■), jute (□).

The adhesion of *S. vacuolatus* to the substrates was also measured in terms of efficiency of the photochemistry of the immobilized algae. Light intensity plays a key role, affecting the

photochemical efficiency of attached algae. All the measured parameters varied at different light intensities, resulting particularly sensitive in the range 50-150 PAR. In this interval, we observed the maximum decrease of photochemical efficiency that declined from the initial values of 0,65 for jute and 0,55 for cotton to 0,48 for jute and 0,44 of cotton (Figure 3).

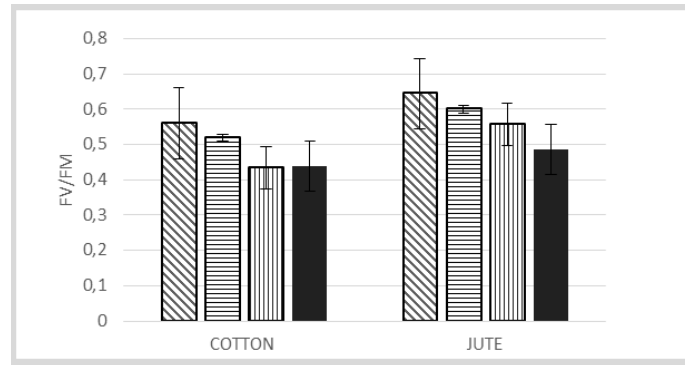


Figure 3. *Fv/Fm* of *S. vacuolatus* cultures on cotton and jute carriers. 2th day (||||), 4th day (≡), 8th day (|||), 16th day (■).

Accordingly, in both type of carriers the microalgae reduced their quantum yield values during the experiment time, with a similar trend, and also in this case higher light intensities showed inhibitory effects on this parameter (Figure 4a, b).

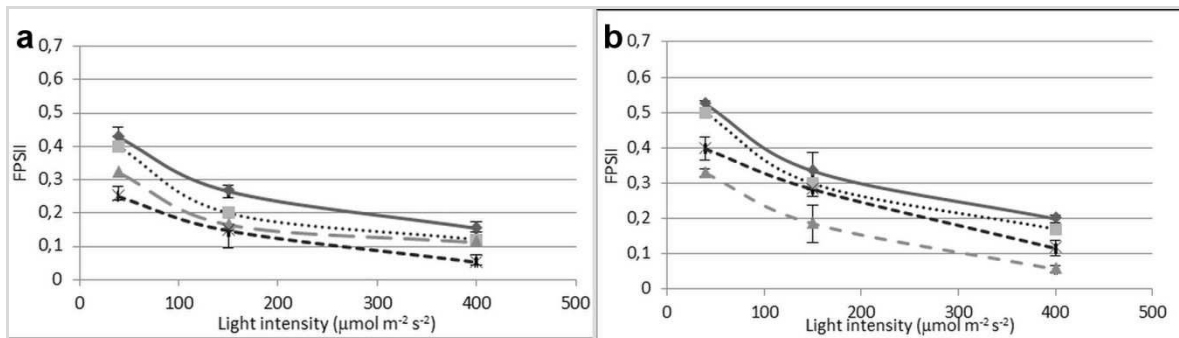


Figure 4. Quantum yield of *S. vacuolatus* cultures grown on cotton (a) or jute (b) carriers. 2th day (—), 4th day (····), 8th day (- - -), 16th day (— —).

In our experiments, also NPQ, a parameter related to the photoprotection of PSII (Lavaud et al., 2000), was scarcely influenced by the type of carrier, and depended mainly on light intensity. NPQ initially achieved high values already at very low light intensity (0,269 at 39 $\mu\text{mol m}^{-2} \text{s}^{-1}$), but in the following days the values decreased to 0,1. At higher light intensities NPQ increased, reaching the maximum values of 0,450 at 480 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

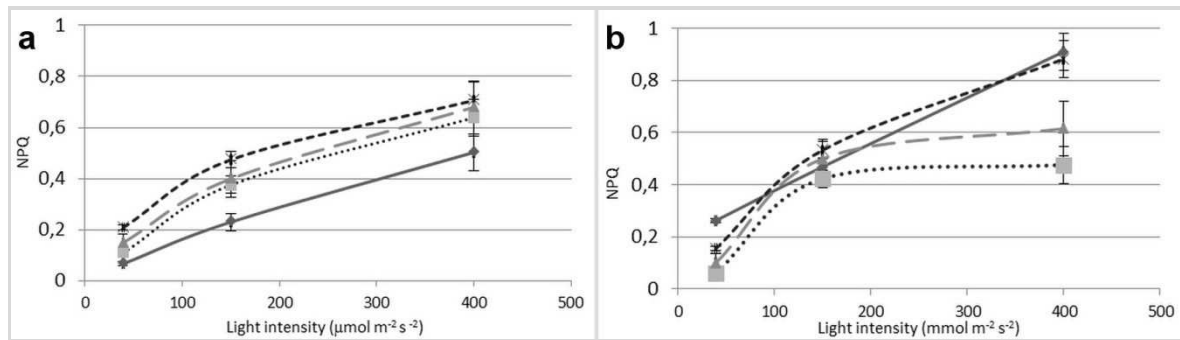


Figure 5. NPQ of *S. vacuolatus* cultures grown on cotton (a) or jute (b) carriers. 2th day (—), 4th day (····), 8th day (- - -), 16th day (- · - ·).

4. Discussions

Immobilized-based systems of microalgal cultivations provide new opportunities for reducing costs and natural resources consumption, but a systematic study on substrate and strain selection is still required. Recent evidence on algal biofilm cultivation system indicates that natural materials like cotton can sustain the algal growth for several months and *Scenedesmus* is showing a very promising attitude in biofilm-based technologies (Gross and Wen, 2014). On the other hand, this genus is characterized by a high number of species (about 800 taxa have been described, according to Hegewald, (1998)); many *Scenedesmus* strains with unknown biotechnological performances are presently held in the algal collections distributed over the world, and a protocol for a high throughput screening is needed. The laboratory approach proposed in this study has shown promising results in terms of reliability, costs and times required; indeed, both the techniques adopted are not expensive and furnish results in short times. Digital image analysis can represent a tool very effective to record the algal growth on attached surfaces, and the development of measuring systems shows that is possible to furnish a detailed characterization of coloured surfaces (Leon et al., 2006). In a recent study on the colonization of lithic surfaces we have demonstrated that it is possible to investigate the behaviour of different strains of Cyanobacteria and green algae by image analysis and that CLSM microscopy can be a tool to describe in a quantitative way the pioneering attitude of different phototrophic organisms (Marasco et al., 2016). However, this kind of approach requires long times (two months), that represent a limit when it is necessary to gain a quick response on the biotechnological features of a strain. The use of PAM fluorometry coupled with digital image analysis has shown that it is possible provide data also on the physiological status of

the attached algal cells, that plays a fundamental importance for the selection of a proper strain. It is possible to evaluate the algal growth through measurements of fluorescence after dark adaptation, as indicated by von Werder and Venzmer (2013); moreover under controlled conditions carbon fixation and PSII are linearly correlated (Maxwell and Johnson, 2000). The F_v/F_m generally has a maximum value of 0,7/0,8 under optimal conditions (Masojídek et al., 2003), but in our test this parameter never achieved these values. A low F_v/F_m ratio can be due to photoinhibition, usually caused by a synergism between high irradiance and other forms of environmental stress, as temperature extremes, or high dissolved oxygen concentration (Bjorkman and Demmig, 1987). However, the test on carriers are made in absence of water, a stress condition frequently experienced by aeroterrestrial microalgae communities (Häubner et al., 2006), that can account for the reduction F_v/F_m ratio. In our tests, we have obtained the better data of photochemical efficiency during the first four days, even though the extension of colonization remained almost constant over the time course of the test. In the following days, the quantum yield decrease was relevant, and it is known that nitrate depletion can leads to a progressive inactivation of PSII reaction centres (Falkowski, 1992; Parkhill et al., 2001). On the other hand, the enhancement of NPQ, which is considered the main protection mechanism against photooxidative damage of photosynthetic machinery (Horton and Hague 1988), points to a stress condition experienced by algal cells attached to the carriers, that needs further experiments to be fully understood. Our results prompt us to develop a protocol based on image analysis and PAM fluorometry tests lasting seven days: it is not necessary to extend them to the second week. In this way, a very high number of strains and carriers could be assayed in a short time. Much work is necessary to fully develop this screening system, particularly more data on the effect of carriers texture and on biochemical mechanisms of adhesion of algal cells to the surfaces, that should be produced by interdisciplinary studies carried out by an integrated team of biologists, biochemists and engineers.

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CHAPTER FIVE

Growth and biomass productivity of *Scenedesmus vacuolatus* on a twin-layer system and a comparison with suspension-based culture systems.

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Abstract:

Scenedesmus is a genus of microalgae employed for several industrial uses. Industrial cultivations are performed in open ponds or in closed photobioreactors (PBRs). In the last years, a novel type of PBR based on immobilized microalgae has been developed termed porous substrate photobioreactors (PSBR) to achieve significant higher biomass density during cultivation in comparison to classical PBRs. This work presents a study of the growth of *Scenedesmus vacuolatus* strain 053 on a PSBR Twin-Layer System at different light intensities ($600 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ or $1000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$), different types and concentrations of the nitrogen sources (nitrate or urea), and at two CO₂ levels in the gas phase (2% or 0.04% v/v). The microalgal growth was followed by monitoring the attached biomass density as dry weight, the specific growth rate and pigment accumulation. The highest productivity ($29 \text{ g m}^{-2} \text{d}^{-1}$) was observed at a light intensity of $600 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and 2% CO₂. The types and concentrations of nitrogen sources did not influence the biomass productivity. Instead the highest light intensity of $1000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and an ambient CO₂ concentration (0.04%) resulted in a significant decrease of productivity to 18 and 10-12 $\text{g m}^{-2} \text{d}^{-1}$, respectively. When compared to the performance of similar cultivation systems ($15\text{-}30 \text{ g m}^{-2} \text{d}^{-1}$) these results indicate that the twin layer cultivation system is a competitive technique for intensified microalgal cultivation in terms of productivity and, at the same time, biomass density.

Keywords

Scenedesmus vacuolatus, Porous Substrate Bioreactor, Twin-Layer, biomass, pigments.

Introduction

The genus *Scenedesmus* is frequently cultivated at pilot and large scale for numerous biotechnological applications, and different species have been successfully employed: *S. obliquus*, *S. (Halochlorella) rubescens* and *S. dimorphus* for wastewater treatments (Gonzales *et al.* 1997 e, Shi *et al.* 2007, Zhang *et al.* 2014), production of polysaccharides for cosmetics (Liu *et al.* 2010, Castro *et al.* 2012) or fatty acids (Wiltshire *et al.* 2000, Tang *et al.* 2011), *S. almeriensis* for bioactive food ingredients (Granado Lorenzo *et al.* 2009), *S. armatus*, *S. bernardii* and *S. obliquus* for biodiesel production (Kaewkannetra *et al.* 2012), and finally *S. dimorphus*, *S. quadricauda*, and *S. subspicatus* for biomass production and CO₂ mitigation (Goswami *et al.* 2011, Osundeko *et al.* 2013). A large variety of *Scenedesmus* species are continuously tested to assess their biotechnological potential and, given their good growth performance and robustness, their adaptability to novel types of photobioreactors, as e.g. reported for *S. obtusiusculus* and *S. ovalternus* (Koller *et al.* 2017 a, and b). Recently, we have concentrated our efforts on the less known species *S. vacuolatus*, which has shown promising results as a biomass and biofuel producer both in outdoor and indoor cultures (Olivieri *et al.*, 2013; Gargano *et al.* 2016).

Until now, for industrial uses strains of *Scenedesmus* and in general microalgae were grown in suspension cultures and different techniques are employed for this type of cultivation (Borowitzka 1999, Carvalho *et al.* 2006, Ugwu *et al.* 2008). Some of these techniques are based on open cultures and use of natural light (for example raceway-ponds; Jimenez *et al.* 2013, Grobbelaar *et al.* 1985, Hase *et al.* 2000). Others use closed systems such as photobioreactors (PBRs) of different design (e.g., tubular, flat plate, cylindrical tanks) illuminated by artificial light and supplemented with CO₂ (Pirt *et al.* 1983, Posten *et al.* 2009, Chen *et al.* 2011, Tamburic *et al.* 2011, Koller *et al.* 2017 a, and b).

Microalgal cultures in suspension-based systems are subjected to different types of problems (Wiffels *et al.* 2009, Stephens *et al.* 2010, Wang *et al.* 2015) such as contamination in open ponds (McBride 2014), large costs for photobioreactor design, construction and operation (Jorquera *et al.* 2010; Ruiz *et al.*, 2016) and overall low

biomass density (on average $<5 \text{ g L}^{-1}$). (Naumann *et al.* 2013, Wang *et al.* 2015). The bottleneck is the distribution of light in the suspension and the dynamics of cell light-exposure that is responsible for a reduced photosynthesis efficiency (Gargano *et al.* 2015, Carbone *et al.* submitted). In the last years, new types of photobioreactors (PSBRs) based on immobilized microalgae have been developed to resolve the problems of suspension cultivation of microalgae (Ozkan *et al.* 2013, Schulze *et al.* 2014, Blanken *et al.* 2014) ensuring the optimization of the irradiance, and the enhancement of CO_2 transfer (Gross *et al.* 2015). A particularly promising PSBR is the Twin-Layer System. Here, microalgae are attached by self-adhesion on a hydrophilic, sheet-like porous substrate, which separates algal biomass from the bulk of the medium (Nowack *et al.* 2005, Melkonian *et al.* 2010). A Twin Layer allows very high standing crops of algal biomass ($>100 \text{ g L}^{-1}$) and requires low energy for transfer and harvesting (Schulze *et al.* 2015 Podola *et al.* 2017).

However, as for suspended systems, the optimization of biomass productivity and attached density strongly depends on the inputs provided to the system, such as light intensity, and carbon and nitrogen supply. In the literature only few studies deal with the behaviour of *Scenedesmus* spp. on a twin-layer system and these were focused on wastewater treatment (Shi *et al.* 2007, Shi *et al.* 2014), but a characterization and optimization of biomass growth for *Scenedesmus* spp. are missing.

Therefore , in this contribution, the effects of light intensity , CO_2 addition, different nitrogen sources and concentrations in the medium on the growth of the microalga *Scenedesmus vacuolatus* on a Twin-Layer system were studied to evaluate biomass productivity in comparison with experiments previously carried out using the same strain grown in suspension-based cultivation systems.

Scenedesmus vacuolatus was chosen because in a previous experiment shown a good skill of adhesion on different types of carriers in immerse conditions (Carbone *et al.* 2017).

Material and Methods

Microalgal strains and medium

Scenedesmus vacuolatus strain 053 was used. The strain was obtained from the Algal Collection of the University Federico II in Naples “ACUF” (<http://www.acuf.net>). The cultures grew in Bold's Basal Medium (BBM) in Erlenmeyer flasks at $23 \pm 2 \text{ }^\circ\text{C}$ and a light intensity of $20\text{--}40 \text{ } \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ using a light/dark cycle of 14/10 h in a temperature-controlled in growth chamber.

Photobioreactor and operating condition.

Microalgae were grown in a lab-scale Twin-Layer system (TL-PBR). Design details were according to Shi et al.(2007) and Schulze et al. (2015; Figure 1).

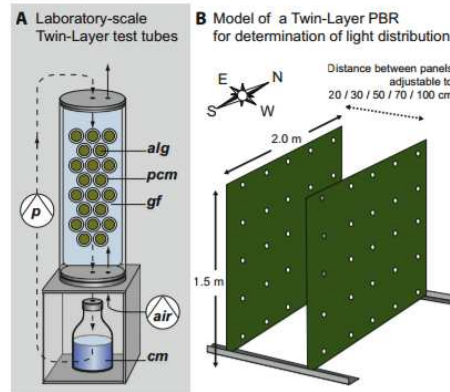


Figure 1. Twin-layer system. A: Laboratory-scale Twin-Layer test tubes. *alg* - immobilized microalgae, *pcm* - polycarbonate membrane as a carrier for microalgae, *gf* - glass fibre mat, *air* - membrane pump for air supply, *cm* - culture medium. B: Model of a 1:1 Twin-Layer photobioreactor for the determination of the diurnal light distribution on the reactor surfaces Figure and legend reproduced from Schultze et al.2015

A sodium discharge lamp (f 2×3 vertically orientated SON-T AGRO 400W, Philips, Hamburg, Germany) was used for provision of light. Two light intensities ($600 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ respectively) were used. Photosynthetic active radiation (PAR) was measured by a quantum sensor (LI-190SA, LI-COR Biosciences GmbH, Bad Homburg, Germany) and the light/dark cycle was set to 14/10 h.

The experiments were carried out in the presence of atmospheric CO_2 (0.4%) or additional CO_2 (2% v/v). In the presence of atmospheric CO_2 , the TL-PBR was aerated by membrane pumps with 0.75 L min^{-1} ambient air, instead the CO_2 was mixed with compressed air to a concentration of 2% (v/v) and the gas flow through the TL-PBR was 1.25 L min^{-1} .

Microalgae were inoculated at density of $5 \text{ g dry weight m}^{-2}$ by filtration onto polycarbonate membranes (PC40, $0.4 \mu\text{m}$ pore size, 25 mm diameter, Whatman, Dassel, Germany) on an area of 2.54 cm^2 .

Culture Medium

For the experiment, different nitrogen sources and concentrations were tested: regular BBM with nitrate (www.ccap.ac.uk), a modified medium with urea in the same concentration (BBM-U), a modified BBM medium with 3 times nitrate concentration (BBM-3N). To avoid nutrient limitation, the medium was replaced every two days. The pH was measured every two days with a pH meter before and after medium replacement (benchtop pH meter Mettler Toledo).

Attached biomass quantification

The polycarbonate discs with the attached algal biomass were harvested every two days in triplicate. Surplus biomass was scraped off with the aid of an 18 mm diameter tube to guarantee that only biomass in the inoculated area would be measured. The samples were lyophilized for two hours and were weighted in an analytic balance (Sartorius Bovenden, Germany). Biomass growth was quantified as attached dry weight biomass density (g m^{-2}) over time.

Pigment characterization

After lyophilization, 1 mg of the biomass was taken off from the disk and was mixed with quartz sand to obtain homogeneous powder.

Chlorophyll a, b and carotenoids were extracted by the DMSO method and were analyzed spectrophotometrically (Shimadzu UV-2450). The equation used for the quantification were:

$$\text{Ch-a} = 12.47A_{665.1} - 3.62A_{649.1}$$

$$\text{Ch-b} = 25.06A_{649.1} - 6.5A_{665.1}$$

$$C_{x+c} = (1000A_{480} - 1.29\text{Ch-a} - 53.78\text{Ch-b})/220$$

where Ch-a, Ch-b and C_{x+c} are the quantities (mg l^{-1}) of chlorophyll a, b and carotenoids respectively, A is the absorbance measured at the specified wavelength in quartz cuvettes. (Hiscox and Israelstam, 1979)

Data analysis

ANOVA was used for statistical analysis, Tukey's test was used as a post hoc test and Minitab 17 was the software program.

Results

In suspension-based systems, under light intensities higher than $250 \mu\text{mol m}^{-2} \text{s}^{-1}$, the photosynthesis activity of *Scenedesmus vacuolatus* became less efficient and also the biomass value decreased (Gargano et al. 2016, Carbone et al. submitted). A similar behavior was observed also in other *Scenedesmus* species (Sforza et al. 2014). However, in the TL-PBR, the irradiance is distributed over a larger surface area, and the upper biofilm layers shade the lower layers thus reducing photoinhibition processes (Gross et al. 2015, Schultze et al. 2015). Based on the results obtained in previous tests carried out with the TL-PBRs (Schultze et al. 2015), in the experiments on *S. vacuolatus* the growth rates and pigment contents were analyzed at two different high light intensities ($600 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and $1000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) in a BBM medium with different nitrogen sources. Moreover the effect of the CO_2 was tested by growing the biofilm under two different carbon dioxide regimes.

Effect of light intensity and CO_2 level on the growth and biomass productivity

Figure 2 reports the data of biomass density expressed as a function of time for two different values of light intensity and in the presence of 2% CO_2 . At $600 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, *S. vacuolatus* achieved a maximum biomass density of 258 g m^{-2} after ten days; this value decreased slightly only on the fourteenth day. The average specific growth rate over the first 8 days was $0,88 \text{ d}^{-1}$. Instead at a light intensity of $1000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, *S. vacuolatus* achieved the maximum biomass density of 119 g m^{-2} after ten days, more significantly decreasing to 80 g m^{-2} at the end of the experiment. Indeed the specific growth rate at $1000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ over the first 8 days was lower ($0,61 \text{ d}^{-1}$). A lag phase of growth of minimum two days was observed under both light conditions (Fig. 2).

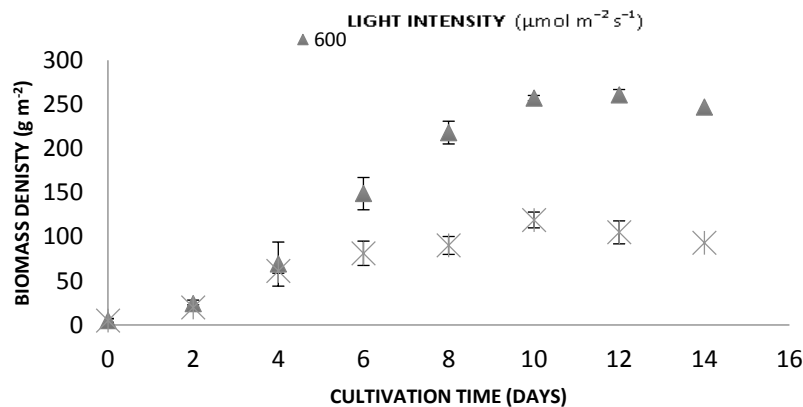


Figure 2. Biomass growth of *Scenedesmus vacuolatus* in the presence of 2% of CO_2 at two different light conditions ($600 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ or $1000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$).

Figure 3 shows the Chl *a* and *b* contents as a function of the time. Their level increased from 0.2 to 5 g m⁻² and from 0.1 to 1.8 g m⁻² respectively during the first ten days, when the biofilm was exposed to 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$, then a slight decrease of Chl *a*, and a more marked reduction of Chl *b* occurred. At the end of the time course of the experiment, the Chl *a* content was about 3.8 g m⁻², while Chl *b* collapsed to 0.1 g m⁻². A similar trend was observed at 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$: the values of chlorophyll *a* and *b* were initially similar (0.247 g m⁻² for chlorophyll *a* and 0.240 g m⁻² for chlorophyll *b*), but after four days Chl *a* reached concentrations three-times higher than Chl *b* (1.9 g m⁻² vs 0.6 g m⁻²). Also in this case after the peak at day ten, the content of both chlorophylls *a* and *b* dropped, with Chl *b* decreasing more significantly (Figure 3).

These variations are probably linked to the availability of the nitrogen source in the medium: under conditions of N-starvation the breakdown of an internal source of nitrogen as e.g. in Chlorophylls furnishes nitrogen for the synthesis of proteins and nucleic acids (Evans 1989).

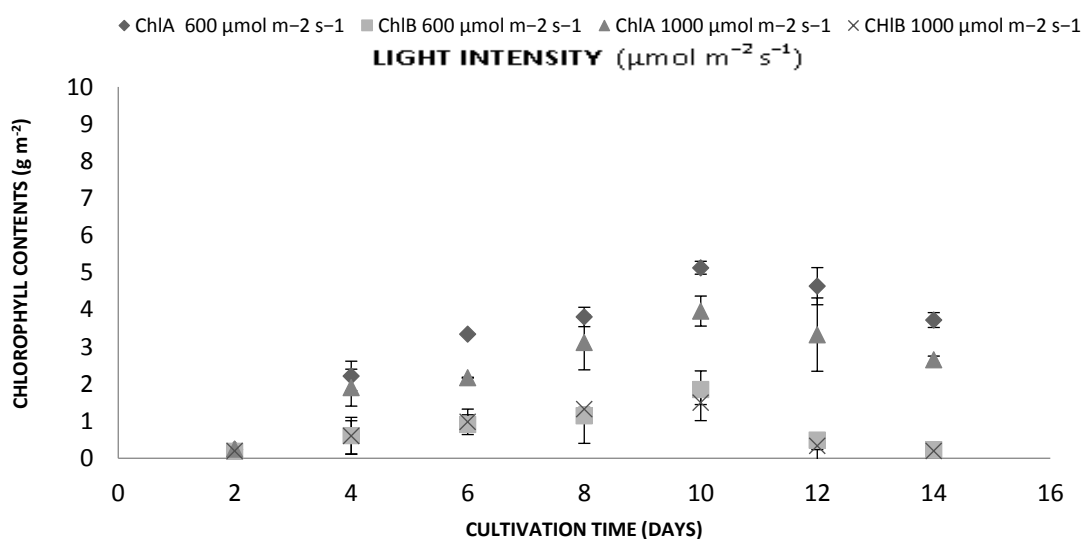


Figure 3. Chlorophyll *a* and *b* concentration of *S. vacuolatus* in the presence of 2% of CO₂ at two light conditions of 600 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ or 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$.

The presence of 2% CO₂ positively influenced the increase of production of biomass and pigments. Indeed, under atmospheric CO₂ the experiment lasted only eight days because at both light intensities tested, algae slipped down from the polycarbonate disks, being unable to create a stable biofilm. Under this condition, the maximum biomass density of *S. vacuolatus* was about 50 g m⁻² (figure 4), irrespective of the light intensity used. Indeed in

both bases the average specific growth rate over the first 6 days was around 0.61. The absence of an effect of light intensity clearly proves that the CO_2 was the limiting factor.

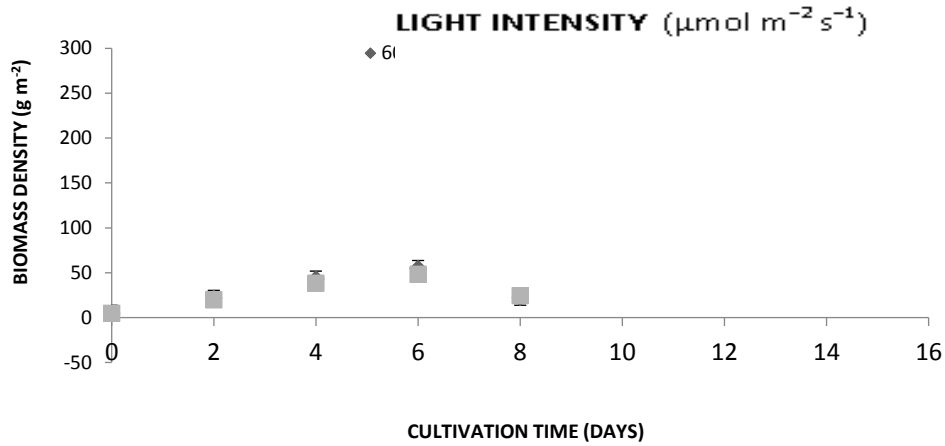


Figure 4. Biomass growth of *S. vacuolatus* in atmospheric CO_2 at two light intensity of $600 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$.

Figure 5 reports that the contents of chlorophyll *a* and *b* over time did not significantly increase under an atmospheric CO_2 regime. Indeed for both the conditions, the maximum content of chlorophyll *a* and *b* was about 1 g m^{-2} after six days.

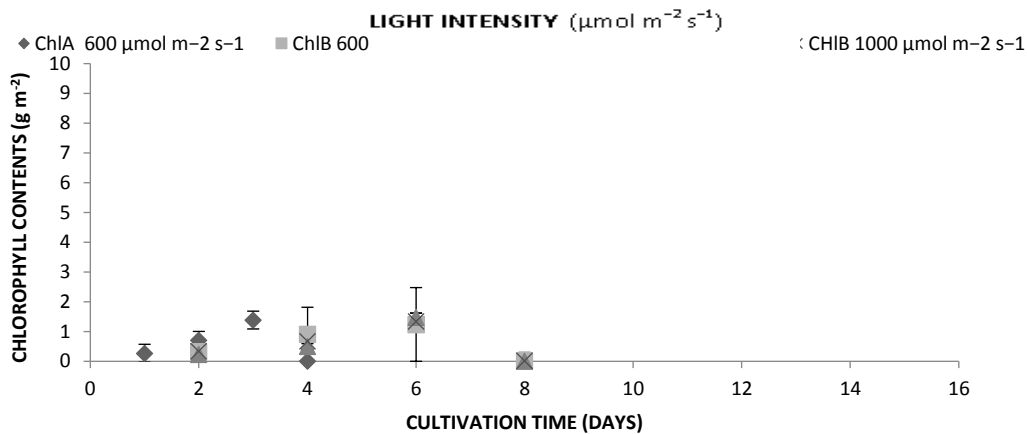


Figure 5 Chlorophyll *a* and *b* of *S. vacuolatus* grown on atmospheric CO_2 at two light irradiances of $600 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$.

Effect of different nitrogen sources on *S. vacuolatus* growth

Next, *S. vacuolatus* was tested at $600 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ on a TL-PBR in the presence of a BBM medium with urea or 3N nitrate, to study their effect on biomass production. As a rule, in suspension culture these medium modifications increase biomass and pigment

production (Arumugan et al. 2013, Soletto et al.2004). The experiments were carried out at $600 \mu\text{mol m}^{-2} \text{s}^{-1}$, in the presence of 2% CO_2 or atmospheric CO_2 .

Under 2% CO_2 , the growth rate in the presence of urea and 3N nitrate was higher than that observed with BBM 1N (3N: 1.1 d^{-1} , urea: 1.0 d^{-1} , BBM 1N: 0.88 d^{-1}). On the other hand, *S. vacuolatus* grown on the three different nitrogen sources achieved comparable maximum biomass yields (urea 274 g m^{-2} , 3N nitrate 266.7 g m^{-2} BBM 1N: 261 g m^{-2} , Figure 6 A), Also Chlorophyll *a* and *b* concentrations were not influenced by the nitrogen sources (figure 6 B).

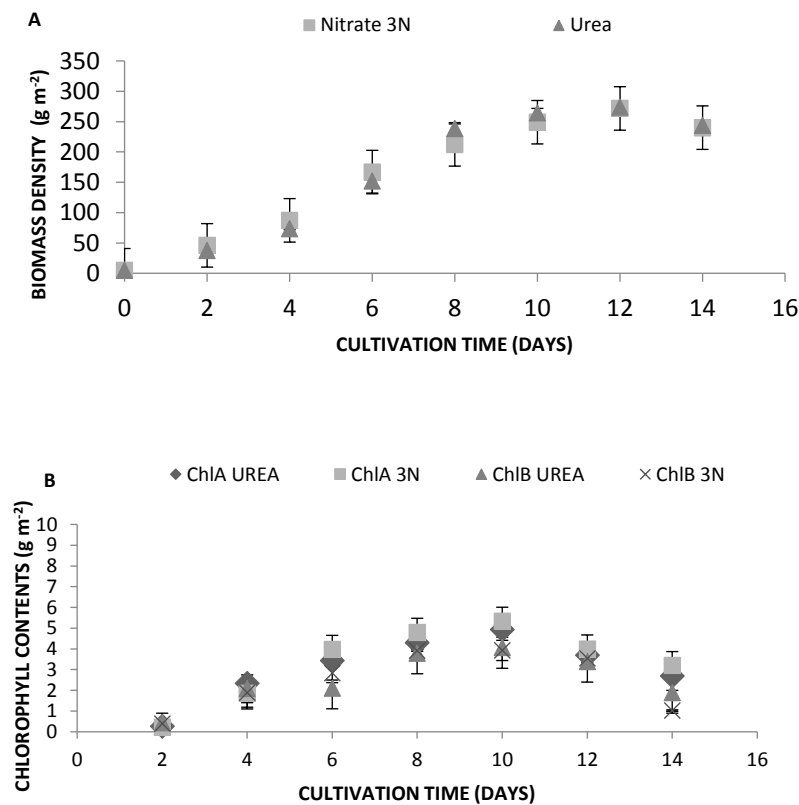


Figure 6. Biomass (Aa) and chlorophyll contents (b) in the presence of 2% CO_2 and under different nitrogen sources.

In the presence of atmospheric CO_2 , the situation was partially different. The experiment was carried out for a shorter time because after eight days the algae slipped down from the polycarbonate disks.

When urea was added as a nitrogen sources the total values of biomass, total pigment concentration and biomass productivity were the same as obtained with BBM medium.

Indeed, the maximum biomass density was around 50 g m^{-2} , chlorophyll *a* and *b* highest values were 1.5 gm^{-2} (Figure 7a).

In the presence of nitrate 3N the maximum values of biomass and chlorophyll contents were significantly higher (Figures 3 and 7). On the other hand, the growth rate was only slightly influenced by nitrate source and concentration (3N 0.89 d^{-1} ; urea and BBM 1N 0.7 d^{-1}).

In all experiments, the carotenoid concentration was scarcely influenced by nitrogen sources. Very low concentration of carotenoids were found (they did not exceed values of 0.2 g m^{-2} in presence of 2% CO_2 and 0.09 g m^{-2} under atmospheric CO_2 . This feature appears also in suspension culture: *Scenedesmus vacuolatus* was tested for the production of secondary carotenoids but it was not a good candidate (Orosa et al.2000).

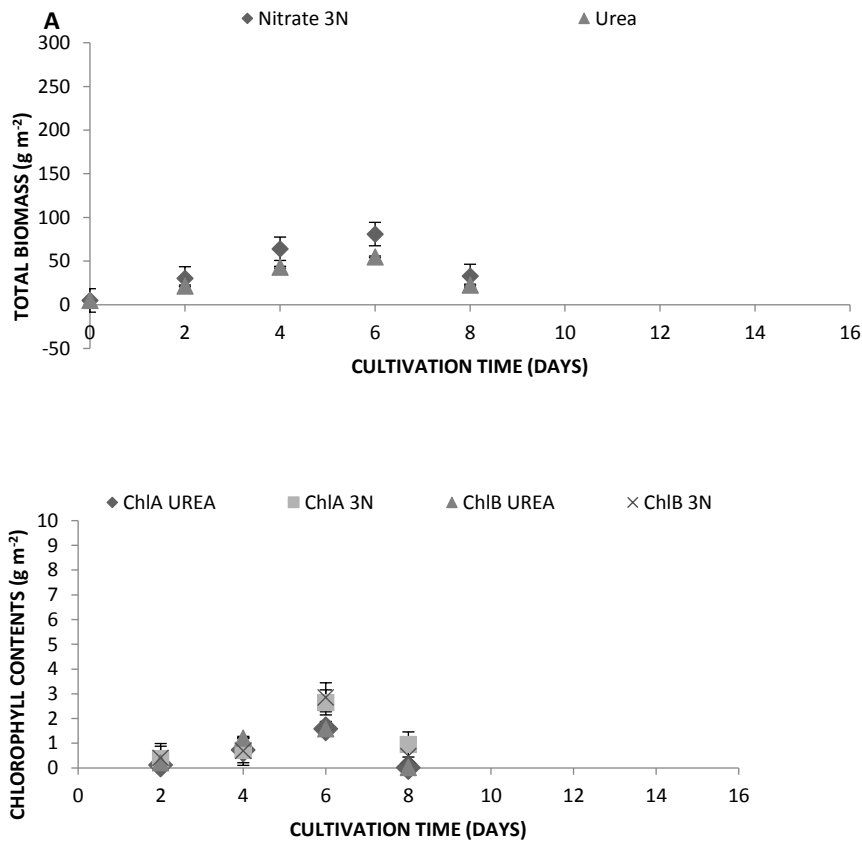


Figure 7. Biomass (a) and chlorophyll contents (b) under , BBM 3N and BBM urea.

Discussion

Our principal results showed that a light intensity of $600 \mu\text{mol m}^{-2} \text{ s}^{-1}$ and addition of 2% CO_2 positively influenced biomass productivity and light yield of *Scenedesmus vacuolatus* in comparison to the other investigated operating conditions (figure 8). Indeed in this type

of condition, *Scenedesmus vacuolatus* achieved a maximum biomass productivity value around $29 \text{ g m}^{-2} \text{ d}^{-1}$ and a maximum light yield around 0.95. This maximum biomass content was obtained regardless of the type and concentration of the nitrogen source in the medium.

Instead when increasing the light intensity up to $1000 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$ still in presence of 2% CO_2 the maximum biomass productivity decreased to $15 \text{ g m}^{-2} \text{ d}^{-1}$ becoming 36% lower than the one obtained at $600 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$ (figure 8). It is plausible to suppose that this negative effect could be due to photoinhibition of the photosystem within the microalgal cells as reported also for suspended cultivation in the literature (Ben-Amotz & Avron 1981; Richmond 1986) Vonshak et al.1992). Consequently this results shows that also immobilized biomass cultivation systems (depending on the algal species used; see Schultze et al. 2015 for a different result, below) may not be able to protect the cells from photoinhibition at very high light intensities.

It is known that the addition of CO_2 positively influences the growth of microalgae culture (e.g. Matsumoto et al.1997). Also our experiments confirmed this for *S. vacuolatus* in a TL-PBR: in the presence of atmospheric CO_2 the biomass productivity and light yield were significantly lower than those obtained in presence of 2% CO_2 (figure 8).

The analysis of the photosynthetic efficiency is also reported in figure 8 as light to biomass yield. When optimised, the results are always between 0.9 and 1 g E^{-1} , whereas in the literature also values lower than 0.5 g E^{-1} can be found such as in the work of Gargano et al. 2016.

The most interesting comparison in figure 8 was with *Halochlorella rubescens* (Schultze et al. 2015) tested on the same type of PSBRs as in our experiment. *Halochlorella rubescens* achieved the best results in presence of 3% CO_2 . The maximum value of biomass productivity at $1000 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$ of this microalga (31 g m^{-2}) was slightly higher than that of *Scenedesmus vacuolatus* whereas the maximum value of light yield (0.6 g E^{-1}) was lower than in our experiments at $600 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$.

These results, when optimized, are promising since they clearly appear competitive with other experiments on similar cultivation system (figure 8): the cultivation of *Scenedesmus vacuolatus* based on a TL-PBR displays very good values of biomass productivity ($> 25 \text{ g m}^{-2} \text{ d}^{-1}$), light yield (close to 1 g E^{-1}). Moreover also as in other experiments, addition of CO_2 is necessary to increase the productivity at moderate or high light intensities

(Blanken et al.2014, Gross et al. 2013, Ozkan et al.2012, Ji et al. 2013, Schultze et al.2015)

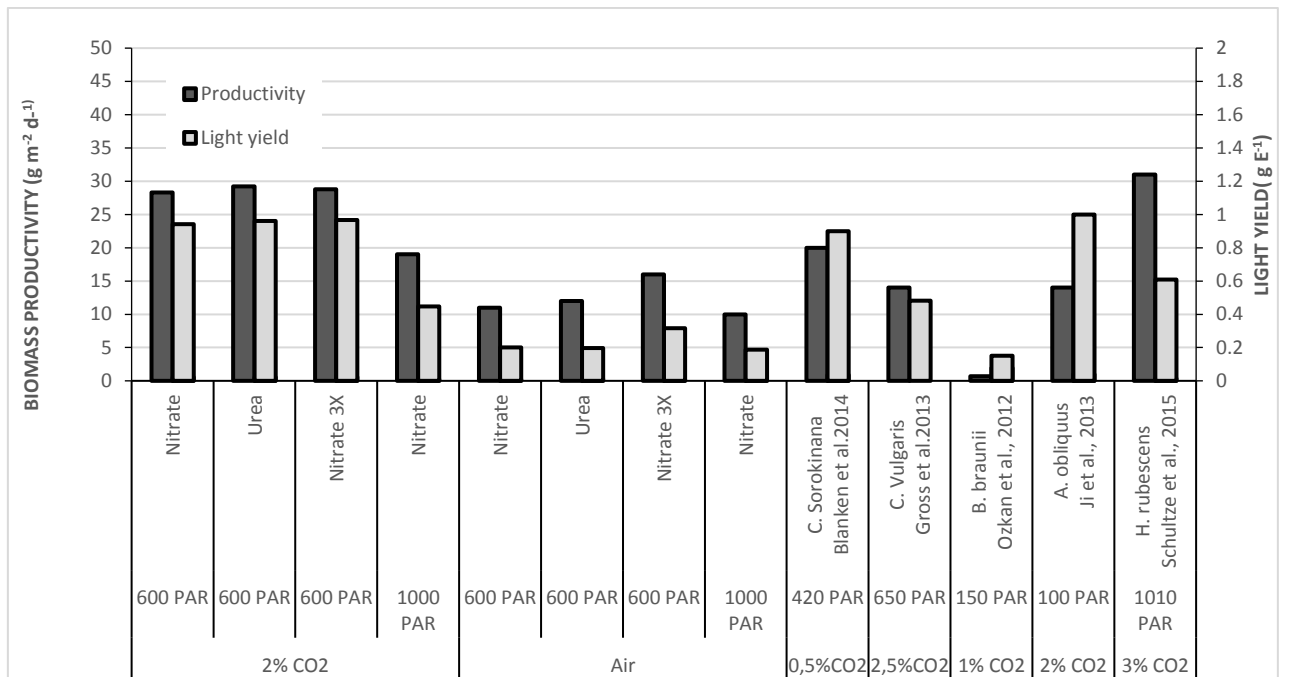


Figure 8. Comparison between the results of maximum biomass productivity and maximum light yield of *Scenedesmus vacuolatus* in this study and the same parameters of other microalgae on PSBRs in other studies.

In suspension culture, *Scenedesmus vacuolatus* showed an interesting potential for biotechnological applications. It has been successfully employed in the biotransformation of sterols, being able to catalyze reactions of hydroxylation, reduction and side chain-degradations (Della Greca et al, 1996, Pollio et al. 1996), and has recently revealed a promising cosmeceutical prospective (Chatzikonstantinou et al., 2016). Indeed in other experiments, the growth of *Scenedesmus vacuolatus* 053 was studied in bubble column photobioreactor on outdoor conditions (Gargano et al.2016) and in a climate chamber (Carbone et al. submitted) at 250 $\mu\text{E m}^{-2} \text{s}^{-1}$. However, when grown outdoors and under indoor light intensities, this microalga achieved a maximum areal biomass productivities and light yields of around 10.4 $\text{g m}^{-2} \text{d}^{-1}$, 0.170 g E^{-1} (Gargano et al. 2016) and 13.4 $\text{g m}^{-2} \text{d}^{-1}$ 0.320 g E^{-1} (Carbone et al., submitted), which are significantly lower than the one reported in this work and at very low biomass concentration ($<3 \text{ g L}^{-1}$). We also observe that in liquid culture *Scenedesmus vacuolatus* was influenced by the concentration of nitrate and in presence of nitrate 3N the values of biomass productivity were higher than those in presence of nitrate sources (Carbone et al. submitted). Also other species of *Scenedesmus* in suspension culture had lower growth performances than in the TL-PBR:

in the experiment of Sforza et al. 2014, *Scenedesmus obliquus* achieved the maximum biomass productivity of 12 g m⁻² and in the experiment of De Morais and Costa the same organism achieved only 1.6 g m⁻².

Conclusions

These preliminary results show that *Scenedesmus vacuolatus* achieves a good biomass and pigment concentrations and encourage further studies using the TL-PBR. In particular it should be interesting to test the production of industrial components of *this microorganism* such as EPS or fatty acids.

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CHAPTER SIX

CONCLUSION

CONCLUSION

In this PhD thesis I show that *Scenedesmus vacuolatus* could be a good candidate for industrial uses and I evaluate different ways for optimizing biomass productivity of this organism in both type of cultivations: liquid and on substrate.

Preliminarily, by analyzing several strains of *Scenedesmus vacuolatus*, I prove the best candidate is 053. It appears to be good source for lipid productivities and POX activity; moreover it shows the best performances of growth in the range of pH and salinity.

To evaluate the problem of biomass productivity in liquid culture of this strain by studying photochemical processes and gas exchange data, I assume the model of Eilers and Peters (1988). This model, as optimized in Gargano et al. 2015 by assessing the kinetic parameters by fluorescence measurements, allows to analyze the photosynthetic process and the pitfalls linked to it. Then I investigate the effect of the most common operating conditions used in massive microalgae cultivations on the photosynthetic performance of *Scenedesmus vacuolatus*.

In case of photobioreactor, I highlight that the bottleneck of the photosynthesis process is photoinhibition. Indeed at high light intensity, the kinetic parameter (in the model) α linked to photon capture is lower than the parameter β linked to photoinhibition. This implies that the photon capture is slower than photoinhibition.

Scenedesmus vacuolatus, moreover, shows a very good growth performances in liquid cultures in enclosed photobioreactors achieving high biomass and chlorophyll concentrations in presence of 2% CO₂.

Then the study was concentrated on culture on solid substrate. This is a recent technology with a lot of new problems. Several works have shown that the type of substrate influenced the microalgae attachment and consequently the biomass productivity (Gross and Wen 2014). So one of the critical factor is the attachment of microalgae on substrate.

For this reason I studied the growth and photosynthetic activity of *Scenedesmus vacuolatus* during the first steps of the adhesion process in submerged condition on two

different types of substrate, cotton and jute by the combination of photograph imaging analysis, which allows to study the growth thanks to the different colour of microalgae on tissueses, and pulse amplitude modulation fluorimetry (pam), which allows to examine the photochemical processes, obtaining data on physiological status of the attached algal cells.

Also in this case the use of *Scenedesmus vacuolatus* shows positive outcomes: it has a good ability of attachment. Indeed already the second day of inoculation on substrate there were the colony of microalgae on substrates and these organisms presented also a good performance of the photochemical activity.

Moreover the adhesion process of *Scenedesmus vacuolatus* is scarcely influenced by the kind of carrier used. However, these results seem in contrast with other works where it has been shown that the cotton is the best material for the formation of biofilm.

Eventually, in detailed research on the growth of *Scenedesmus vacuolatus* on a solid substrate by evaluating the increase of biomass productivity on a particular type of porous substrate bioreactor (the Twin Layer system), the performances of *Scenedesmus vacuolatus* in liquid systems were lower than that one of attached cultivation though also in attached cultivation the addition of CO₂ is necessary to increase the productivity. In presence of atmospheric CO₂ this organis slides down from the twin layer after one week.

Indeed the maximum areal biomass productivity and light yield of *Scenedesmus vacuolatus* culture in bubble column photobioreactor on outdoor conditions (Gargano et al.2016) and on climate chamber, didn't achieved the values obtained in Twin layer system.

Moreover the *Scenedesmus vacuolatus* culture growth on Twin Layer system shows other different behaviours than those in liquid culture.

Firstly, the Twin layer system needs light intensity more higher than that one in liquid culture because, in the attached cultivation, the irradiance is distributed over a larger surface area reducing photoinhibition processes (Gross et al. 2015).

Secondly, on the contrary of behaviour in liquid culture, the growth of *Scenedesmus vacuolatus* isn't influenced by the type of medium.

Comparison with data obtained from other organisms in other experiments on PSBRs shows that immobilized cultivation of *Scenedesmus vacuolatus* based on Twin-layer system allows to achieve very good values of biomass productivity and light yield and

confirms that also in attached cultivation *Scenedesmus vacuolatus* is a good candidate for industrial uses.

On balance, all obtained results show it should be interesting to test the performance of *Scenedesmus vacuolatus* for production of industrial components such as Eps or FAMES and not only the simple biomass productivity.

Moreover, they induce to think up different ways to optimize the microalgae cultivation.

More precisely, to resolve the highlighted problem of photoinhibition in liquid culture, a new optimized structure of photobioreactors is in planning stage. Here, the photobioreactor is covered by a photochromic photosensitive panel. The panel is able to reduce the light intensity at the fixed value. In this way the circadian clock, characteristic of microalgae, is preserved and the light is never so high to inhibit the photosynthetic process.

Regarding the attached cultivation, it would be interesting to analyze the first steps of microalgae growth also on others type of substrates such as polycarbonate. Also in this type of study, models could be used to discover the pitfalls of attachment process.

Finally about solid porous bioreactor, the use of a horizontal structure could probably avoid the sliding down of the organism in presence of atmospheric CO₂ and increase the biomass.

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